Table 1. Peptide candidates used for personalized peptide vaccine (PPV).

Peptide Name	HLA Restriction	Original Protein	Position	Amino Acid Sequence
CypB-129	A2 A3sup	Cyclophilin B	129-138	KLKHYGPGWV
EGFR-800	A24	EGF-R	800-809	DYVREHKDNI
EZH2-735	A24	EZH2	735-743	KYVGIEREM
HNRPL-140	A2	HNRPL	140-148	ALVEFEDVL
HNRPL-501	A2 A26	HNRPL	501-510	NVLHFFNAPL
Lck-90	A3sup	p56 lck	90-99	ILEQSGEWWK
Lck-208	A24	p56 lck	208-216	HYTNASDGL
Lck-246	A2	p56 lck	246-254	KLVERLGAA
Lck-422	A2 A3sup	p56 lck	422-430	DVWSFGILL
Lck-449	A3sup	p56 lck	449-458	VIQNLERGYR
Lck-486	A24	p56 lck	486-494	TFDYLRSVL
Lck-488	A24	p56 lck	488-497	DYLRSVLEDF
MAP-432	A2 A26	ppMAPkkk	432-440	DLLSHAFFA
MRP3-503	A24	MRP3	503-511	LYAWEPSFL
MRP3-1293	A24	MRP3	1293-1302	NYSVRYRPGL
PAP-213	A24	PAP	213-221	LYCESVHNF
PAP-248	A3sup	PAP	248-257	GIHKQKEKSR
PSA-248	A24	PSA	248-257	HYRKWIKDTI
PSMA-624	A24	PSMA	624-632	TYSVSFDSL
PTHrP-102	A24	PTHrP	102-111	RYLTQETNKV
SART2-93	A24	SART2	93-101	DYSARWNEI
SART2-161	A24	SART2	161-169	AYDFLYNYL
SART3-109	A24 A3sup A26	SART3	109-118	VYDYNCHVDL
SART3-302	A2	SART3	302-310	LLQAEAPRL
SART3-309	A2	SART3	309-317	RLAEYQAYI
SART3-511	A3sup	SART3	511-519	WLEYYNLER
SART3-734	A3sup	SART3	734-742	QIRPIFSNR
UBE-43	A2	UBE2V	43-51	RLQEWCSVI
UBE-85	A2	UBE2V	85-93	LIADFLSGL
WHSC2-103	A2 A3sup A26	WHSC2	103-111	ASLDSDPWV
WHSC2-141	A2	WHSC2	141-149	ILGELREKV

A3sup: HLA-A3 supertype (A3, A11, A31, or A33); EGF-R: Epidermal Growth Factor Receptor; EZH2: enhancer of zeste homolog 2; HNRPL: heterogeneous nuclear ribonucleoprotein L; ppMAPkkk: partial putative mitogen-activated protein kinase kinase kinase; MRP3: multidrug resistance-associated protein 3; PAP: Prostatic acid phosphatase; PSA: prostate specific antigen; PSMA: Prostate specific membrane antigen; PTHrP : parathyroid hormone-related peptide; SART2: squamous cell carcinoma antigen recognized by T cells 2; SART3: squamous cell carcinoma antigen recognized by T cells 3; UBE2V: ubiquitin-conjugated enzyme variant Kua; WHSC2: Wolf-Hirschhorn syndrome candidate 2.

the selected peptides are administered weekly for at least the first cycle of six vaccinations, since a clear trend toward better immune responses was observed among the patients who underwent the weekly administration protocol compared to those who underwent a bi-weekly protocol in our previous clinical trials [47].

One of the noticeable characteristics of our PPV formulation is that it screens vaccine antigen candidates before vaccination, based on CTL-precursor frequencies and/or immunoglobulin G (IgG) titers specific to each of the candidates in pre-vaccination blood samples from each patient [25]. In the earlier stage of translational studies of PPV, pre-existing immunity was defined by the frequencies of CTL precursors in pre-vaccination peripheral blood mononuclear cells (PBMC) by detecting peptide-specific IFN-γ production by enzyme-linked immunosorbent assay (ELISA) [47-51]. However, we are currently evaluating the pre-existing im-

munity to vaccine candidates by measuring peptide-specific IgG titers in pre-vaccination plasma by the multiplex bead-based Luminex assay rather than CTL precursor frequencies, since the performance characteristics, such as the sensitivity and reproducibility, of the current T-cell assays are sometimes unsatisfactory for detecting low frequencies of antigen-specific CTL [52, 53]. In contrast to the drawbacks inherent to T-cell assays, the multiplex bead-based Luminex technology that we have developed to monitor B-cell responses allows simple, quick, and highly reproducible high-throughput screening and monitoring of IgG responses specific to a large number of peptide antigens with a tiny amount of plasma [36, 54, 55]. Indeed, the selection of vaccine antigens based on IgG titers seemed to be useful for predicting CTL boosting after vaccination in our clinical trials. The predictive power of evaluating the existence of antigen-specific CTL precursors solely by the humoral responses before vaccination could be estimated at around 50% when four peptides were chosen for PPV in each patient [56, 57].

2.3. Clinical Trials of PPV for Advanced Cancers

A series of phase I, I/II, and II clinical trials of PPV has been conducted in the past several years for various types of advanced cancer patients. Table **2** summarizes the immune and clinical responses of advanced cancer patients treated with PPV. In the following sections, we provide some detailed information on these clinical studies.

2.3.1. Castration-Resistant Prostate Cancer (CRPC)

In phase I studies of PPV for advanced HLA-$A2^+$ or HLA-$A24^+$ CRPC, we have reported increased cellular and humoral immune responses and decreased PSA levels in some patients [58, 59]. In a phase I dose-escalation study of PPV (1, 3, and 5 mg/peptide injection) for HLA-$A24^+$ CRPC, we have also demonstrated that a dose of 3 mg/peptide injection showed better cellular immune responses to vaccine peptides than either 1 or 5 mg/peptide injections, although the maximum tolerated dose (MTD) was not determined [56]. In addition, in a phase I/II study of 58 HLA-$A2^+$ or HLA-$A24^+$ CRPC patients, a combination of PPV and low-dose estramustine phosphate (EMP) showed a median survival time (MST) of 17 months (95% confidence interval (95% CI), 12 to 25 months), along with a decreased serum PSA level in the majority (76%) of patients [60]. The same study also revealed that fewer lymphocytes, negative immunological responses to vaccine antigens, and poor performance status were independent predictors of disease-related death [60].

Subsequently, we conducted a randomized phase II trial to compare PPV plus low-dose EMP with standard-dose EMP in HLA-$A2^+$ or HLA-$A24^+$ CRPC patients. The patients receiving PPV in combination with low-dose EMP showed a significantly longer progression-free survival [MST, 8.5 months vs 2.8 months; hazard ratio (HR), 0.28 (95% CI, 0.14-0.61); $P = 0.0012$] and overall survival [MST, undefined vs 16.1 months; HR, 0.30 (95% CI, 0.1-0.91); $P = 0.0328$] than those receiving standard-dose EMP alone, suggesting the efficacy of this combination therapy [61]. In another phase II study, we compared docetaxel-based chemotherapy (DBC)-resistant CRPC patients undergoing PPV (n =20) with a historical control (n = 17). MSTs from the failure of previous DBC treatments were 17.8 and 10.5 months in patients treated with and without PPV, respectively [62]. These promising results suggested that PPV warrants further study as a novel therapy for CRPC patients, even for those with progressive disease following DBC treatment. A phase III randomized clinical trial of PPV is currently under way in DBC-resistant CRPC patients.

2.3.2. Malignant Glioma

In a phase I clinical study, we demonstrated the feasibility of PPV for HLA-$A2^+$ or HLA-$A24^+$ advanced malignant glioma patients [47]. The clinical responses of 27 patients who received more than six vaccinations were partial response (PR) in 5, stable disease (SD) in 8, and progressive disease (PD) in 8 patients, with a MST of 20.7 months. Significant levels of IgG specific to vaccine peptides were detected after vaccination in the tumor cavity or spinal fluid obtained from patients who had shown favorable clinical responses. Another phase I clinical trial in HLA-$A24^+$ patients with recurrent or progressive GBM also showed the safety and increased immune boosting of PPV with potential clinical benefits, with a MST of 10.6 months even after failure of the standard temozolomide treatment [57]. On the basis of these promising results, double-blind randomized phase III trials are under way in GBM patients resistant to the standard treatment.

2.3.3. Pancreatic Cancer and Biliary Tract Cancer

We have conducted a phase I trial of PPV in 13 HLA-$A2^+$ or HLA-$A24^+$ patients with advanced pancreatic cancer, where the patients were treated by PPV at three different doses (1, 2, or 3 mg/peptide) in combination with gemcitabin (GEM) [63]. This combination therapy was well tolerated, and 11 of 13 patients (85%) showed reduced tumor sizes and/or levels of tumor markers. Peptide-specific CTL responses were augmented at each dose level, and the increment of peptide-specific IgG antibodies was dependent on the peptide dose. These findings suggested that GEM did not inhibit the immune responses induced by PPV. Subsequently, we conducted a phase II trial of PPV in combination with GEM to evaluate the safety, clinical efficacy, and antigen-specific immune responses as a front-line therapy for 21 HLA-$A2^+$ or HLA-$A24^+$ nonresectable patients with advanced pancreatic cancer [64]. This combination therapy was also well tolerated, and the best clinical responses were PR in 7, SD in 9, and PD in 5 patients. The MST of all 21 patients was 9 months with a 1-year survival rate of 38%, which was better than that reported for GEM alone (MST of 5.7 months with a 1-year survival rate of 18%) [65]. Importantly, the MST was 15 months in patients who showed immunological responses to vaccine peptides.

We also conducted a phase II clinical trial of PPV in 25 HLA-$A2^+$ or HLA-$A24^+$ chemotherapy-resistant patients with advanced biliary tract cancer [66]. When two to four vaccine peptides selected by pre-existing immunity were administered to the patients in this study, humoral and/or T-cell responses specific to the vaccine antigens were substantially induced in a subset of the patients without severe adverse events. Greater numbers of selected and vaccinated peptides were significantly favorable factors for overall survival (HR = 0.258, 95% CI = 0.098-0.682, $P = 0.006$) in this study (Table **3**).

Table 2. List of clinical trials of personalized peptide vaccines (PPV) for advanced cancer.

Organ	Disease condition	Phase of trial	HLA restriction	Combined treatment	No. of Patients	Clinical response	MST (months)	Toxicities (Grade 3/4)	Humoral response (%)	Cellular response (%)	Reference
Prostate (CRPC)	Advanced	I	A24	-	10	SD 50%	NA	-	60	40	[58]
Prostate (CRPC)	Advanced	I	A24	EMP	13	PR 63%	24	G3, 5%	91	55	[114]
Prostate (CRPC)	Advanced	I	A2	-	10	SD 30%	22	-	70	40	[59]
Prostate (CRPC)	Advanced	I/II	A24	EMP	16	PR 43%	17	-	50	71	[115]
Prostate (CRPC)	Advanced	I/II	A2/A24	EMP	58	PR 24%	17	G3, 7%	88	78	[60]
Prostate (CRPC)	Advanced	I	A24	EMP	15	PR 13%	24	-	47	67	[56]
Prostate (CRPC)	Advanced	II (Randomized)	A2/A24	EMP	57	8.5M vs 2.8M (PFS)	22.4M vs 16.1M	-	64	50	[61]
Prostate (CRPC)	Advanced	II	A2/A24/ A3sup/A26	-	42	PR 12%	17.8	-	44	34	[62]
Prostate	Localized	II	A24	-	10	PR 20%	NA	-	80	80	[116]
Brain	Advanced malignant glioma	I	A2/A24	-	21	PR 24%, SD 38%	NA	-	40 - 64	50 - 82	[47]
Brain	Advanced GBM	I	A24	-	12	PR 17%, SD 42%	10.6	-	17	75	[57]
Pancreas	Advanced	I	A2/A24	GEM	13	PR 15%, SD 54%	7.6	-	69	69	[63]
Pancreas	Advanced	II	A2/A24	GEM	21	PR 33%, SD 43%	9	-	72	78	[64]
Biliary tract	Advanced	II	A2/A24/ A3sup/A26	Chemo-therapy	25	SD 32%	NA	G3, 4%	35	47	[66]
Stomach	Advanced	I	A2/A24	-	13	SD 45%	NA	-	80	50	[67]
Stomach Colorectal	Advanced	I/II	A2/A24	S-1	11	SD 36%	NA	G3, 18%	81	63	[69]
Colorectal	Advanced	I	A24	-	10	PR 10%	NA	-	70	50	[68]
Colorectal	Metastatic	I	A2/A24	UFT UZEL	13	SD 43%	19.6	G3, 7.7%	69	85	[70]
Lung	Advanced	I	A24	-	10	SD 80%	15.2	-	40	40	[50]
Lung	Advanced (NSCLC)	II	A2/A24 A3sup/A26	Chemo-therapy	41	SD 56%	10.1	G3, 7%	49	34	[71]
Lung	Advanced (SCLC)	II	A2/A24 A3sup/A26	Chemo-therapy	10	SD 20%	6.2	G3, 4%	83	83	[72]

(Table 2) contd....

Organ	Disease condition	Phase of trial	HLA restriction	Combined treatment	No. of Patients	Clinical response	MST (months)	Toxicities (Grade 3/4)	Humoral response (%)	Cellular response (%)	Reference
Urothelial	Advanced	I	A2/A24	-	10	CR 10%, PR 10%	24	-	80	80	[73]
Kidney	Metastatic	I	A2/A24	-	10	SD 60%	23	-	80	5	[74]
Uterine, Ovary	Recurrent	I	A2/A24	-	14	SD 36%	NA	G3, 8%	86	86	[49]
Skin	Malignant melanoma	I	A2/A24	-	7	SD 43%	NA	-	57	86	[51]

CRPC: castration-resistant prostate cancer; GBM: glioblastoma multiforme; SCLC: small cell lung cancer; NSCLC: non-small cell lung cancer; A3sup: HLA-A3 supertype (A3, A11, A31, or A33); EMP: estramustine phosphate; GEM: gemcitabine; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; PFS: progression-free survival; MST: median survival time; NA: not assessed; M: months.

Table 3. Biomarkers for personalized peptide vaccines (PPV) for advanced cancer.

Type of cancer	Factor	Statistical analysis (HR, 95% CI, P value)	Reference
Miscellaneous (n = 500)[a]	Performance status (1, 2, 3 vs 0) Lymphocyte counts (<1500μL vs > 1500μL) IgG responses to antigens after vaccination (no vs yes)	HR = 2.295; 95% CI, 1.653 - 3.188; P < 0.0001 HR = 1.472; 95% CI, 1.099 - 1.972, P = 0.0095 HR = 1.455; 95% CI, 1.087 - 1.948, P = 0.0116	[36]
Prostate (CRPC, n = 40)	IL-6 MDSC	(Not determined) (Not determined)	[81]
Non-small lung cell cancer (n =41) [a]	C-reactive protein (CRP)	HR = 10.115, 95% CI = 2.447 – 41.806, P = 0.001	[71]
Biliary tract (n = 25)[a]	IL-6 Albumin Numbers of vaccine peptides	HR = 1.123, 95% CI = 1.008 -1.252, P = 0.035 HR = 0.158; 95% CI, 0.029 - 0.860; *P* = 0.033 HR = 0.258, 95% CI = 0.098-0.682, *P* = 0.006	[66]

[a]Potential biomarkers for PPV were determined by multivariate Cox regression analyses. ; HR: hazard ratio; CI: confidence interval; CRPC: castration-resistant prostate cancer; MDSC: myeloid-derived suppressor cells.

2.3.4. Gastric Cancer and Colorectal Cancer

In a phase I clinical trial of PPV in 13 HLA-$A2^+$ or HLA-$A24^+$ patients with advanced gastric cancer (9 nonscirrhous and 4 scirrhous), prolonged survival was observed in patients who showed cellular and humoral immune responses to the vaccine peptides in the post-vaccination blood samples, including all 4 patients with the scirrhous type [67]. In addition, a phase I clinical trial of PPV in 10 HLA-$A24^+$ patients with advanced colorectal cancer showed one PR and one SD, each continuing for more than 6 months [68].

In a phase I/II clinical trial of PPV in combination with three different doses (20, 40, or 80 mg/m^2/day) of oral administration of a 5-fluorouracil derivative, S-1, for 11 HLA-$A2^+$ or HLA-$A24^+$ advanced gastric or colorectal cancer patients [69], the combined administration of the standard dose (80 mg/m^2/day) of S-1 did not inhibit immunological responses to vaccine antigens, but instead maintained or augmented them. In another phase I clinical trial for 13 HLA-$A2^+$ or HLA-$A24^+$ metastatic colorectal cancer patients [70], the combined treatment of PPV and the oral administration of a 5-fluorouracil derivative, UFT, and calcium folinate, UZEL, proved to be safe and to induce good antigen-specific immune responses. In this trial, IgG responses to the vaccine peptides correlated well with overall survival. These encouraging results suggest that combined treatment with PPV and standard chemotherapeutic agents might be promising for advanced gastric and colorectal cancers.

2.3.5. Lung Cancer

The prognosis of advanced lung cancer patients remains very poor, with a MST of around 6-10 months. Phase I and II studies of PPV in a small number of patients with refractory NSCLC demonstrated that PPV was safe and well tolerated,

with no major adverse effects, and that PPV treatment resulted in longer survival (MST of 10.1 or 15.2 months) [50, 71]. A clinical study in 10 advanced small cell lung cancer (SCLC) also showed the safety and feasibility of PPV [72].

2.3.6. Urothelial Cancer

A phase I clinical trial of PPV was conducted in 10 HLA-$A2^+$ or HLA-$A24^+$ refractory urothelial cancer patients [73]. In this study, some patients treated by PPV showed clear clinical responses as evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST) criteria with boosted immune responses: CR in 1, PR in 1, and SD in 2 patients. These 4 responders showed better progression-free survival (MST, 21 months) and overall survival (MST, 24 months), suggesting the potential clinical efficacy of PPV for advanced urothelial cancer.

2.3.7. Other Cancers

We also conducted phase I clinical trials for other advanced cancers, including metastatic renal cell carcinoma (RCC) [74], gynecologic cancers [49], and malignant melanoma [51]. All of these studies demonstrated that PPV was safe and well tolerated with no major adverse effects, and that good immune responses to vaccine antigens were induced in many of the patients after PPV. Further clinical trials would be required to clearly prove the clinical benefits of PPV in these cancers.

2.4. Biomarkers for PPV (Table 3)

Recent clinical trials of cancer immunotherapies, including peptide-based cancer vaccines, have demonstrated that only a subset of patients show clinical benefits. Furthermore, unexpectedly, some large clinical trials in the past several years have demonstrated that cancer vaccines might sometimes show worse clinical outcomes [75, 76]. It would thus be important to identify predictive biomarkers that could accurately assess anti-tumor immune responses and predict patient prognosis following the administration of cancer vaccines. In some clinical trials, several post-vaccination biomarkers, including CTL responses, Th1 responses, delayed-type hypersensitivity (DTH), and autoimmunity, have been reported to be associated with clinical responses.[77-80]. However, there are currently no validated biomarkers for cancer vaccines in widespread use.

To identify biomarkers for PPV, we statistically reviewed 500 advanced cancer patients undergoing PPV from October 2000 to October 2008 [36]. Both lymphocyte counts before vaccination (P = 0.0095) and increased IgG response (P = 0.0116) to the vaccine peptides after vaccination, along with performance status (P < 0.0001), were well correlated with overall survival. In CRPC patients treated with PPV (n = 40), a comprehensive study of soluble factors assessed by multiplexed bead array in plasma and gene expression profiles by DNA microarray in PBMC demonstrated that higher IL-6 level and granulocytic myeloid-derived suppressor cells (MDSC) in the peripheral blood before vaccination were closely related to poorer prognosis in the vaccinated patients [81]. By multivariate Cox regression analyses in patients with refractory NSCLC (n = 41), higher C-reactive protein (CRP) level before vaccination was a significant predictor of unfavorable overall survival (HR = 10.115, 95% CI = 2.447 – 41.806, P = 0.001) [71]. In addition, in refractory biliary tract cancer patients (n = 25), multivariate Cox regression analyses showed that higher IL-6 and lower albumin levels before vaccination were significantly unfavorable factors for overall survival [HR = 1.123, 95% CI = 1.008 - 1.252, P = 0.035; HR = 0.158, 95% CI = 0.029 - 0.860, P = 0.033; respectively] [66].

Collectively, these findings suggested that less inflammation may contribute to better responses to PPV, indicating that the evaluation of inflammatory factors before vaccination could be useful for selecting cancer patients who are appropriate for PPV (Table **3**). An early phase clinical trial is under way to reveal whether or not the blockage of IL-6-mediated inflammatory signaling with a humanized anti-IL-6 receptor monoclonal antibody, tocilizumab, would be beneficial for enhancing the immune and/or clinical responses after PPV in advanced cancer patients who show higher plasma IL-6 levels [82, 83].

3. OTHER NEW TYPES OF PEPTIDE VACCINES

Recent early phase clinical trials have also demonstrated significant advances in other types of therapeutic peptide-based vaccines [19, 20]. Several new types of peptide-based vaccines are reviewed in this section (Fig. **1**).

3.1. Multi-Peptide Vaccine Consisting of CTL and Helper T-Cell Epitopes

Numerous helper T-cell epitopes have been identified from TAA. Since helper T cells are known to play crucial roles in the efficient induction of CTL responses, cancer vaccines, which consist of both HLA class II-restricted helper epitopes recognized by CD4 T cells and class I-restricted CTL epitopes recognized by CD8 T cells, have been developed and clinically tested [84-89]. For example, Kuball *et al.* conducted a phase I study of a multi-peptide vaccine consisting of multiple CTL epitopes from Wilms tumor gene-1 (WT-1), proteinase 3 (Pr3) and mucin 1 (MUC1), and MUC1-helper epitope or pan HLA-DR epitope (PADRE) [84]. Each peptide was formulated separately and injected at a different site. In this study, an increase in PADRE-specific CD4 T cells, which appeared unable to produce IL2, was observed after vaccination, and regulatory T cells were increased, suggesting that helper epitope peptides have the potential to induce not only helper T cells but also regulatory T cells. Krug *et al.* tested the safety and immunogenicity of a WT1 vaccine comprised of four class I and class II-restricted peptides in patients with malignant pleural mesothelioma or NSCLC expressing WT1 [85]. They showed that this multivalent WT1 peptide vaccine induced both CD4 and CD8 T-cell responses in a high proportion of patients with minimal toxicity.

3.2. Multi-Peptide Cocktail Vaccine

If each of multiple peptides are formulated separately and injected at a separate site, the number of peptides employed for vaccination might be limited. One strategy for overcoming this limitation is to generate multi-peptide cocktail vaccines, since one preparation could contain more than 10 different peptides. Although the issue of competition between

individual peptides to bind to HLA molecules on the APCs still remains [46], different types of multi-peptide cocktail vaccines have been developed; vaccines consisting of CTL epitope peptides alone [90, 91] or those of both CTL epitope and helper epitope peptides [86-89].

Barve *et al.* conducted a phase I/II study of a multi-peptide cocktail vaccine, IDM-2101, consisting of nine CTL epitope peptides and the PADRE helper epitope peptide with Montanide ISA51 in patients with metastatic non-small cell lung cancer [86]. No significant adverse events were noted except for low-grade erythema and pain at the injection site. One-year survival in the treated patients was 60%, with a median overall survival of 17.3 months. One complete response (CR) patient was observed in the total of 63 patients. Slingluff *et al.* conducted a multicenter randomized trial to examine the immunogenicity of a multi-peptide cocktail vaccine containing 12 melanoma-associated HLA class I-restricted peptides (12MP) for $CD8^+$ T cells and tetanus peptide or a mixture of six melanoma-associated helper peptides (6MHP) for $CD4^+$ T cells in the presence or absence of cyclophosphamide pretreatment in 167 patients with resected stage IIB to IV melanoma [87]. However, the combination of 6MHP with 12MP paradoxically reduced the circulating $CD8^+$ T-cell response, and cyclophosphamide pretreatment had no measurable effect on $CD8^+$ or $CD4^+$ responses. Clinical outcome was not improved by adding melanoma-associated helper peptides or by adding cyclophosphamide.

Rammensee and his colleagues also reported a phase I/II trial of a multi-peptide cocktail vaccine, which consisted of 13 synthetic peptides (11 HLA-A*0201-restricted CTL epitopes and 2 helper epitopes derived from prostate tumor antigens) for 19 HLA-$A2^+$ hormone-sensitive prostate cancer patients with biochemical recurrence after primary surgical treatment [88]. The vaccine was well tolerated, and stabilized or slowed down PSA progress in 4 of the 19 patients. The same group also developed another cocktail vaccine, IMA901, which consisted of nine HLA-A*0201-restricted CTL epitopes and one helper epitope from renal cell cancer antigens with hepatitis B virus epitope as a marker peptide, for advanced renal cell cancer [89]. In a randomized phase II trial with a single dose of cyclophosphamide, the number of regulatory T cells was reduced, and immune responses to the vaccine peptides were associated with longer overall survival. A randomized phase III study to determine the clinical benefit of IMA901 is ongoing.

3.3. Hybrid Peptide Vaccine

Peptides used in most clinical trials for peptide-based vaccines possess native amino acid sequences with or without slight modification in anchor amino acids to increase their binding capability to HLA molecules. However, hybrid-type peptide vaccines, which use a new artificial peptide fusing two or more peptides, have been devised. For example, the Ii-Key/HER-2/neu hybrid peptide vaccine, a fusion peptide made up of the Ii-Key 4-mer peptide and HER-2/neu (776-790) helper epitope peptide, has been reported [92, 93]. The Ii/Key 4-mer peptide is the shortest active sequence of the Ii protein, which catalyzes direct charging of MHC class II epitopes to the peptide-binding groove, circumventing the need for intracellular epitope processing [94]. Phase I studies of the Ii-Key/HER-2/neu hybrid peptide vaccine in patients with prostate cancer showed that this vaccine is safe and can induce HER-2/neu-specific cellular immune responses in vaccinated patients [93]. In addition, significant decreases in circulating regulatory T-cell frequencies, plasma HER2/neu, and serum TGF-beta levels were observed.

Nishimura *et al.* reported an artificially synthesized helper/killer-hybrid epitope long peptide (H/K-HELP) of MAGE-A4 cancer antigen [95]. In the first case report, a patient with pulmonary metastasis of colon cancer was vaccinated with MAGE-A4-H/K-HELP in combination with OK432 and Montanide ISA51. There were no severe side effects except for a skin reaction at the injection site. Vaccination with MAGE-A4-H/K-HELP induced MAGE-A4-specific Th1 and Tc1 immune responses and the production of MAGE-A4-specific complement-fixing IgG antibodies. Tumor growth and tumor markers were significantly decreased in this patient.

3.4. Long Peptide Vaccine

The classical types of peptide vaccines have consisted of short epitope peptides with minimal optimal lengths, which are recognized by CTLs or helper T cells in an HLA class I- or class II-restricted manner, respectively. However, direct binding of short peptides to nonspecific cells without a co-stimulatory capacity has been reported to bear the potential to induce tolerance to antigen-specific T cells rather than to induce their activation in some mouse models [39-41]. Therefore, a novel approach using synthetic long peptides, which need to be taken up by professional APCs and processed for presentation by HLA class I and/or class II molecules, has been developed for cancer vaccination, although the efficiency and mechanisms of presentation of exogenous long peptides in human HLA class I remain to be fully elucidated [96]. Synthetic long peptides may contain not only HLA class I-restricted but also HLA class II-restricted epitopes, which can activate helper T cells important for the efficient induction of antigen-specific CTL responses.

Several clinical studies using a pool of multiple synthetic long peptides have been reported, since a mixture of multiple synthetic long peptides is likely to contain multiple HLA class I-restricted and class II-restricted T-cell epitopes, which could be applicable to any patients irrespective of their HLA types [42-45, 97-100]. Melief and his colleagues showed that a vaccine composed of a synthetic long peptide pool derived from high-risk-type human papillomavirus (HPV)-16 E6/E7 oncoproteins successfully induced HPV-specific immune responses [42, 43]. They conducted a phase I study of HPV16 E6 and E7 overlapping long peptides in end-stage cervical cancer patients [42]. Cocktails of nine E6 peptides and/or four E7 peptides covering the entire sequences of E6 and E7 proteins showed a strong and broad T-cell response dominated by immunity against E6 after four subcutaneous administrations with Montanide ISA51 at 3-week intervals. Subsequently, they conducted a phase II study of the same vaccine in patients with HPV-positive grade 3 vulvar intraepithelial neoplasia, which is a chronic disorder caused by HPV [43]. At 3 months after the last vaccination, 12 of 20 patients (60%) had clinical responses and reported relief of symptoms. Five women had complete regression of the lesions. At 12 months of follow-up, 15 of 19

patients (79%) had clinical responses, with a complete response in 9 of 19 patients (47%).

The same group also reported a synthetic long peptide vaccine targeted for p53. This p53 synthetic long peptide vaccine (p53-SLP) consisted of 10 synthetic 25-mer to 30-mer long overlapping peptides, spanning amino acids 70–248 of the wild-type p53 protein. In a phase I/II trial of the p53-SLP vaccine in 10 patients with metastatic colorectal cancer, p53-specific T-cell responses were induced in 9 of 10 patients as measured by IFN-γ enzyme-linked immunospot (ELISPOT), proliferation, and cytokine bead arrays [97]. Subsequently, a phase II study of the same vaccine in 20 ovarian cancer patients with recurrent elevation of CA-125 showed that SD, as determined by CA-125 levels and CT scans, was observed in 2 out of 20 patients (10%) as the best clinical response, but no relationship was found between the clinical response and vaccine-induced immunity [44]. IFN-γ–producing p53-specific responses were induced in CD4 T cells, but not in CD8 T cells, in all patients who received four immunizations. The absence of p53-specific CD8 T-cell responses might be attributable to the dominant production of Th2 cytokines by CD4 T cells, which have inhibitory effects on CTL induction. Nevertheless, the combined use of p53-SLP vaccine and a low dose of cyclophosphamide or IFN-α has recently been reported to efficiently induce more IFN-γ–producing p53-specific T cells, suggesting that these combinations may potentiate the immunogenicity of the p53-SLP vaccine [98, 99].

Kakimi *et al.* also conducted a phase I trial of an NY-ESO-1 synthetic long peptide vaccine. A 20-mer peptide spanning from amino acid 91 to 110 of NY-ESO-1, called NY-ESO-1f, which includes multiple epitopes recognized by antibodies and CD4 and CD8 T cells, was administered along with OK-432 and Montanide ISA51 to patients with advanced cancers [100]. Both antigen-specific CD4 and CD8 T-cell responses, as well as antibody responses, were increased in 9 of 10 patients.

3.5. Novel Approach for Targeting Peptides to Professional APCs

The goal of cancer immunotherapy is to induce and amplify functional antigen-specific immune responses in order to develop long-lasting immunological memory specific to tumor cells [101, 102]. However, one hurdle to the use of peptide-based vaccines is that the uptake and/or presentation of vaccine peptides by nonspecific cells, but not by professional APCs, leads to CTL anergy through insufficient stimulation [103]. For efficient priming and activation of antigen-specific CTL through vaccination, sufficient amounts of antigens should be presented to T cells by functionally activated, professional APCs for sufficient periods of time [104-107]. In this respect, a novel delivery system for peptide vaccines remains to be developed.

For example, nanotechnology-based antigen delivery has been developing as a vaccine strategy due to its dose-sparing and prolonged antigen presentation features [108, 109]. In particular, polymeric nanoparticles (NP) have attracted increasing attention as carriers of therapeutic immunogens [110]. Antigen peptides encapsulated in polymeric NP are shown to be directly and specifically delivered to professional APCs via phagocytosis without proteolytic degradation, and efficiently cross-presented to induce strong T-cell immunity, whereas those in solution that are internalized by APCs via macropinocytosis are reported to be poorly presented as peptides in complex with MHC class I molecules on cell surfaces [111, 112]. Indeed, we have demonstrated the feasibility of NP consisting of a biodegradable, biocompatible copolymer, poly(D,L-lactide-co-glycolide) (PLGA) carrying antigenic peptides and a toll-like receptor 4 agonist, monophosphoryl lipid A, to efficiently induce CTL responses against TAA in murine tumor models [113]. To increase the efficacy of peptide-based vaccines, such a novel antigen delivery system remains to be developed and clinically examined.

CONCLUSIONS

In the field of cancer immunology and immunotherapy, excitement and enthusiasm have risen around the latest approvals of immunotherapy-based treatments in various cancer types. However, several issues remain to be addressed in order to achieve further development of cancer vaccines. In particular, in view of the complexity and diversity of tumor cell characteristics and host immune cell repertoires, the selection of vaccine peptides appropriate for individual patients based on the pre-existing host immunity before vaccination could be critical for the efficient induction of beneficial antitumor responses in cancer patients. In a series of clinical trials, we have demonstrated promising results of PPV as a new treatment modality for patients with various types of advanced cancer. Further randomized phase III clinical trials are essential to validate the clinical benefits of PPV. Moreover, novel biomarkers for selecting patients who would benefit most from PPV remain to be addressed.

CONFLICT OF INTEREST

Akira Yamada is an Executive Officer for Green Peptide Company, Ltd. Kyogo Itoh received a research grant from the Green Peptide Company, Ltd. and owns stock in the Green Peptide Company, Ltd.

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REFERENCES

[1] Finn, O.J. Cancer immunology. *N. Engl. J. Med.*, **2008**, *358*(25), 2704-2715.

[2] Rosenberg, S.A. Cell transfer immunotherapy for metastatic solid cancer-what clinicians need to know. *Nat. Rev. Clin. Oncol.*, **2011**, *8*(10), 577-585.

[3] Mellman, I.; Coukos, G.; Dranoff, G. Cancer immunotherapy comes of age. *Nature*, **2011**, *480*(7378), 480-489.

[4] Schlom, J. Therapeutic cancer vaccines: current status and moving forward. *J. Natl. Cancer Inst.*, **2012**, *104*(8), 599-613.

[5] Itoh, K.; Yamada, A.; Mine, T.; Noguchi, M. Recent advances in cancer vaccines: an overview. *Jpn. J. Clin. Oncol.*, **2009**, *39*(2), 73-80.

[6] Kantoff, P.W.; Higano, C.S.; Shore, N.D.; Berger, E.R.; Small, E.J.; Penson, D.F.; Redfern, C.H.; Ferrari, A.C.; Dreicer, R.; Sims, R.B.; Xu, Y.; Frohlich, M.W.; Schellhammer, P.F. IMPACT Study

Investigators. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N. Engl. J. Med.*, **2010**, *363*(5), 411-422.

[7] Hodi, F.S.; O'Day, S.J.; McDermott, D.F.; Weber, R.W.; Sosman, J.A.; Haanen, J.B.; Gonzalez, R.; Robert, C.; Schadendorf, D.; Hassel, J.C.; Akerley, W.; van den Eertwegh, A.J.; Lutzky, J.; Lorigan, P.; Vaubel, J.M.; Linette, G.P.; Hogg, D.; Ottensmeier, C.H.; Lebbé, C.; Peschel, C.; Quirt, I.; Clark, J.I.; Wolchok, J.D.; Weber, J.S.; Tian, J.; Yellin, M.J.; Nichol, G.M.; Hoos, A.; Urba, W.J. Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.*, **2010**, *363*(8), 711-723.

[8] Topalian, S.L.; Hodi, F.S.; Brahmer, J.R.; Gettinger, S.N.; Smith, D.C.; McDermott, D.F.; Powderly, J.D.; Carvajal, R.D.; Sosman, J.A.; Atkins, M.B.; Leming, P.D.; Spigel, D.R.; Antonia, S.J.; Horn, L.; Drake, C.G.; Pardoll, D.M.; Chen, L.; Sharfman, W.H.; Anders, R.A.; Taube, J.M.; McMiller, T.L.; Xu, H.; Korman, A.J.; Jure-Kunkel, M.; Agrawal, S.; McDonald, D.; Kollia, G.D.; Gupta, A.; Wigginton, J.M.; Sznol, M. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.*, **2012**, *366*(26), 2443-2454.

[9] Brahmer, J.R.; Tykodi, S.S.; Chow, L.Q.; Hwu, W.J.; Topalian, S.L.; Hwu, P.; Drake, C.G.; Camacho, L.H.; Kauh, J.; Odunsi, K.; Pitot, H.C.; Hamid, O.; Bhatia, S.; Martins, R.; Eaton, K.; Chen, S.; Salay, T.M.; Alaparthy, S.; Grosso, J.F.; Korman, A.J.; Parker, S.M.; Agrawal, S.; Goldberg, S.M.; Pardoll, D.M.; Gupta, A.; Wigginton, J.M. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.*, **2012**, *366*(26), 2455-2465.

[10] van der Bruggen, P.; Traversari, C.; Chomez, P.; Lurquin, C.; De Plaen, E.; Van den Eynde, B.; Knuth, A.; Boon, T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*, **1991**, *254*(5038), 1643-1647.

[11] Türeci, O.; Sahin, U.; Schobert, I.; Koslowski, M.; Scmitt, H.; Schild, H.J.; Stenner, F.; Seitz, G.; Rammensee, H.G.; Pfreundschuh, M. The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40. *Cancer Res.*, **1996**, *56*(20), 4766-4772.

[12] Cheever, M.A.; Allison, J.P.; Ferris, A.S.; Finn, O.J.; Hastings, B.M.; Hecht, T.T.; Mellman, I.; Prindiville, S.A.; Viner, J.L.; Weiner, L.M.; Matrisian, L.M. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin. Cancer Res.*, **2009**, *15*(17), 5323-5337.

[13] Singh-Jasuja, H.; Emmerich, N.P.; Rammensee, H.G. The Tübingen approach: identification, selection, and validation of tumor-associated HLA peptides for cancer therapy. *Cancer Immunol. Immunother.*, **2004**, *53*(3), 187-195.

[14] Kessler, J.H.; Melief, C.J. Identification of T-cell epitopes for cancer immunotherapy. *Leukemia*, **2007**, *21*(9), 1859-1874.

[15] Hu, X.; Chakraborty, N.G.; Sporn, J.R.; Kurtzman, S.H.; Ergin, M.T.; Mukherji, B. Enhancement of cytolytic T lymphocyte precursor frequency in melanoma patients following immunization with the MAGE-1 peptide loaded antigen presenting cell-based vaccine. *Cancer Res.*, **1996**, *56*(11), 2479-2483.

[16] Rosenberg, S.A.; Yang, J.C.; Restifo, N.P. Cancer immunotherapy: moving beyond current vaccines. *Nat. Med.*, **2004**, *10*(9), 909-915.

[17] Purcell, A.W.; McCluskey, J.; Rossjohn, J. More than one reason to rethink the use of peptides in vaccine design. *Nat. Rev. Drug Discov.*, **2007**, *6*(5), 404-414.

[18] Schwartzentruber, D.J.; Lawson, D.H.; Richards, J.M.; Conry, R.M.; Miller, D.M.; Treisman, J.; Gailani, F.; Riley, L.; Conlon, K.; Pockaj, B.; Kendra, K.L.; White, R.L.; Gonzalez, R.; Kuzel, T.M.; Curti, B.; Leming, P.D.; Whitman, E.D.; Balkissoon, J.; Reintgen, D.S.; Kaufman, H.; Marincola, F.M.; Merino, M.J.; Rosenberg, S.A.; Choyke, P.; Vena, D.; Hwu, P. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N. Engl. J. Med.*, **2011**, *364*(22), 2119-2127.

[19] Yamada, A.; Sasada, T.; Noguchi, M.; Itoh, K. Next-generation peptide vaccines for advanced cancer. *Cancer Sci.*, **2013**, *104*(1), 15-21.

[20] Perez, S.A.; von Hofe, E.; Kallinteris, N.L.; Gritzapis, A.D.; Peoples, G.E.; Papamichail, M.; Baxevanis, C.N. A new era in anticancer peptide vaccines. *Cancer*, **2010**, *116*(9), 2071-2080.

[21] Schreiber, R.D.; Old, L.J.; Smyth, M.J. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*, **2011**, *331*(6024), 1565-1570.

[22] Bei, R.; Scardino, A. TAA polyepitope DNA-based vaccines: a potential tool for cancer therapy. *J. Biomed. Biotechnol.*, **2010**, *2010*, 102758.

[23] Yewdell, J.W.; Bennink, J.R. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.*, **1999**, *17*, 51-88.

[24] Chen, W.; McCluskey, J. Immunodominance and immunodomination: critical factors in developing effective CD8+ T-cell-based cancer vaccines. *Adv. Cancer Res.*, **2006**, *95*, 203-247.

[25] Itoh, K.; Yamada, A. Personalized peptide vaccines: a new therapeutic modality for cancer. *Cancer Sci.*, **2006**, *97*(10), 970-976.

[26] Mochizuki, K.; Sato, Y.; Tsuda, N.; Shomura, H.; Sakamoto, M.; Matsuura, K.; Ushijima, K.; Maeda, Y.; Katagiri, K.; Yamada, A.; Todo, S.; Kamura, T.; Harada, M.; Itoh, K. Immunological evaluation of vaccination with pre-designated peptides frequently selected as vaccine candidates in an individualized peptide vaccination regimen. *Int. J. Oncol.*, **2004**, *25*(1), 121-131.

[27] Pilla, L.; Rivoltini, L.; Patuzzo, R.; Marrari, A.; Valdagni, R.; Parmiani, G. Multipeptide vaccination in cancer patients. *Expert Opin. Biol. Ther.*, **2009**, *9*(8), 1043-1055.

[28] Nakao, M.; Shichijo, S.; Imaizumi, T.; Inoue, Y.; Matsunaga, K.; Yamada, A.; Kikuchi, M.; Tsuda, N.; Ohta, K.; Takamori, S.; Yamana, H.; Fujita, H.; Itoh, K. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. *J. Immunol.*, **2000**, *164*(5), 2565-2574.

[29] Shichijo, S.; Nakao, M.; Imai, Y.; Takasu, H.; Kawamoto, M.; Niiya, F.; Yang, D.; Toh, Y.; Yamana, H.; Itoh, K. A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. *J. Exp. Med.*, **1998**, *187*(3), 277-288.

[30] Harashima, N.; Tanaka, K.; Sasatomi, T.; Shimizu, K.; Miyagi, Y.; Yamada, A.; Tamura, M.; Yamana, H.; Itoh, K.; Shichijo, S. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. *Eur. J. Immunol.*, **2001**, *31*(2), 323-332.

[31] Yamada, A.; Kawano, K.; Koga, M.; Matsumoto, T.; Itoh, K. Multidrug resistance-associated protein 3 is a tumor rejection antigen recognized by HLA-A2402-restricted cytotoxic T lymphocytes. *Cancer Res.*, **2001**, *61*(17), 6459-6466.

[32] Harada, M.; Kobayashi, K.; Matsueda, S.; Nakagawa, M.; Noguchi, M.; Itoh, K. Prostate-specific antigen-derived epitopes capable of inducing cellular and humoral responses in HLA-A24+ prostate cancer patients. *Prostate*, **2003**, *57*(2), 152-159.

[33] Inoue, Y.; Takaue, Y.; Takei, M.; Kato, K.; Kanai, S.; Harada, Y.; Tobisu, K.; Noguchi, M.; Kakizoe, T.; Itoh, K.; Wakasugi, H. Induction of tumor specific cytotoxic T lymphocytes in prostate cancer using prostatic acid phosphatase derived HLA-A2402 binding peptide. *J. Urol.*, **2001**, *166*(4), 1508-1513.

[34] Kobayashi, K.; Noguchi, M.; Itoh, K.; Harada, M. Identification of a prostate-specific membrane antigen-derived peptide capable of eliciting both cellular and humoral immune responses in HLA-A24+ prostate cancer patients. *Cancer Sci.*, **2003**, *94*(7), 622-627.

[35] Yoshida, K.; Noguchi, M.; Mine, T.; Komatsu, N.; Yutani, S.; Ueno, T.; Yanagimoto, H.; Kawano, K.; Itoh, K.; Yamada, A. Characteristics of severe adverse events after peptide vaccination for advanced cancer patients: Analysis of 500 cases. *Oncol. Rep.*, **2011**, *25*(1), 57-62.

[36] Noguchi, M.; Mine, T.; Komatsu, N.; Suekane, S.; Moriya, F.; Matsuoka, K.; Yutani, S.; Shichijo, S.; Yamada, A.; Toh, U.; Kawano, K.; Azuma, K.; Uemura, H.; Okuno, K.; Matsumoto, K.; Yanagimoto, H.; Yamanaka, R.; Oka, M.; Todo, S.; Sasada, T.; Itoh, K. Assessment of immunological biomarkers in patients with advanced cancer treated by personalized peptide vaccination. *Cancer Biol. Ther.*, **2011**, *10*(12), 1266-1279.

[37] Avogadri, F.; Merghoub, T.; Maughan, M.F.; Hirschhorn-Cymerman, D.; Morris, J.; Ritter, E.; Olmsted, R.; Houghton, A.N.; Wolchok, J.D. Alphavirus replicon particles expressing TRP-2 provide potent therapeutic effect on melanoma through activation of humoral and cellular immunity. *PLoS One*, **2010**, *5*(9), e12670.

[38] Hong, S.; Qian, J.; Li, H.; Yang, J.; Lu, Y.; Zheng, Y.; Yi, Q. CpG or IFN-α are more potent adjuvants than GM-CSF to promote anti-tumor immunity following idiotype vaccine in multiple myeloma. *Cancer Immunol. Immunother.*, **2012**, *61*(4), 561-571.

[39] Melief, C.J.; van der Burg, S.H. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat. Rev. Cancer*, **2008**, *8*(5), 351-360.

[40] Bijker, M.S.; van den Eeden, S.J.; Franken, K.L.; Melief, C.J.; Offringa, R.; van der Burg, S.H. CD8+ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. *J. Immunol.*, **2007**, *179*(8), 5033-5040.

[41] Toes, R.E.; Offringa, R.; Blom, R.J.; Melief, C.J.; Kast, W.M. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc. Natl. Acad. Sci. USA*, **1996**, *93*(15), 7855-7860.

[42] Kenter, G.G.; Welters, M.J.; Valentijn, A.R.; Lowik, M.J.; Berends-van der Meer, D.M.; Vloon, A.P.; Drijfhout, J.W.; Wafelman, A.R.; Oostendorp, J.; Fleuren, G.J.; Offringa, R.; van der Burg, S.H.; Melief, C.J. Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. *Clin. Cancer Res.*, **2008**, *14*(1), 169-177.

[43] Kenter, G.G.; Welters, M.J.; Valentijn, A.R.; Lowik, M.J.; Berends-van der Meer, D.M.; Vloon, A.P.; Essahsah, F.; Fathers, L.M.; Offringa, R.; Drijfhout, J.W.; Wafelman, A.R.; Oostendorp, J.; Fleuren, G.J.; van der Burg, S.H.; Melief, C.J. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N. Engl. J. Med.*, **2009**, *361*(19), 1838-1847.

[44] Leffers, N.; Lambeck, A.J.; Gooden, M.J.; Hoogeboom, B.N.; Wolf, R.; Hamming, I.E.; Hepkema, B.G.; Willemse, P.H.; Molmans, B.H.; Hollema, H.; Drijfhout, J.W.; Sluiter, W.J.; Valentijn, A.R.; Fathers, L.M.; Oostendorp, J.; van der Zee, A.G.; Melief, C.J.; van der Burg, S.H.; Daemen, T.; Nijman, H.W. Immunization with a P53 synthetic long peptide vaccine induces P53-specific immune responses in ovarian cancer patients, a phase II trial. *Int. J. Cancer*, **2009**, *125*(9), 2104-2113.

[45] Welters, M.J.; Kenter, G.G.; Piersma, S.J.; Vloon, A.P.; Löwik, M.J.; Berends-van der Meer, D.M.; Drijfhout, J.W.; Valentijn, A.R.; Wafelman, A.R.; Oostendorp, J.; Fleuren, G.J.; Offringa, R.; Melief, C.J.; van der Burg, S.H. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. *Clin. Cancer Res.*, **2008**, *14*(1), 178-187.

[46] Rosenberg, S.A.; Sherry, R.M.; Morton, K.E.; Yang, J.C.; Topalian, S.L.; Royal, R.E.; Kammula, U.S.; Restifo, N.P.; Hughes, M.S.; Schwarz, S.L.; Ngo, L.T.; Mavroukakis, S.A.; White, D.E. Altered CD8(+) T-cell responses when immunizing with multiepitope peptide vaccines. *J. Immunother.*, **2006**, *29*(2), 224-231.

[47] Yajima, N.; Yamanaka, R.; Mine, T.; Tsuchiya, N.; Homma, J.; Sano, M.; Kuramoto, T.; Obata, Y.; Komatsu, N.; Arima, Y.; Yamada, A.; Shigemori, M.; Itoh, K.; Tanaka, R. Immunologic evaluation of personalized peptide vaccination for patients with advanced malignant glioma. *Clin. Cancer Res.*, **2005**, *11*(16), 5900-5911.

[48] Hida, N.; Maeda, Y.; Katagiri, K.; Takasu, H.; Harada, M.; Itoh, K. A simple culture protocol to detect peptide-specific cytotoxic T lymphocyte precursors in the circulation. *Cancer Immunol. Immunother.*, **2002**, *51*(4), 219-228.

[49] Tsuda, N.; Mochizuki, K.; Harada, M.; Sukehiro, A.; Kawano, K.; Yamada, A.; Ushijima, K.; Sugiyama, T.; Nishida, T.; Yamana, H.; Itoh, K.; Kamura, T. Vaccination with predesignated or evidence-based peptides for patients with recurrent gynecologic cancers. *J. Immunother.*, **2004**, *27*(1), 60-72.

[50] Mine, T.; Gouhara, R.; Hida, N.; Imai, N.; Azuma, K.; Rikimaru, T.; Katagiri, K.; Nishikori, M.; Sukehiro, A.; Nakagawa, M.; Yamada, A.; Aizawa, H.; Shirouzu, K.; Itoh, K.; Yamana, H. Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients. *Cancer Sci.*, **2003**, *94*(6), 548-556.

[51] Tanaka, S.; Harada, M.; Mine, T.; Noguchi, M.; Gohara, R.; Azuma, K.; Tamura, M.; Yamada, A.; Morinaga, A.; Nishikori, M.; Katagiri, K.; Itoh, K.; Yamana, H.; Hashimoto, T. Peptide vaccination for patients with melanoma and other types of cancer based on pre-existing peptide-specific ctotoxic T-lymphocyte precursors in the periphery. *J. Immunother.*, **2003**, *26*(4), 357-366.

[52] Sharma, P.; Wagner, K.; Wolchok, J.D.; Allison, J.P. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. *Nat. Rev. Cancer*, **2011**, *11*(11), 805-812.

[53] Whiteside, T.L. Immune monitoring of clinical trials with biotherapies. *Adv. Clin. Chem.*, **2008**, *45*, 75-97.

[54] Komatsu, N.; Shichijo, S.; Nakagawa, M.; Itoh, K. New multiplexed flow cytometric assay to measure anti-peptide antibody: a novel tool for monitoring immune responses to peptides used for immunization. *Scand. J. Clin. Lab. Invest.*, **2004**, *64*(6), 535-545.

[55] Mine, T.; Sato, Y.; Noguchi, M.; Sasatomi, T.; Gouhara, R.; Tsuda, N.; Tanaka, S.; Shomura, H.; Katagiri, K.; Rikimaru, T.; Shichijo, S.; Kamura, T.; Hashimoto, T.; Shirouzu, K.; Yamada, A.; Todo, S.; Itoh, K.; Yamana, H. Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing, peptide-specific cellular responses. *Clin. Cancer Res.*, **2004**, *10*(3), 929-937.

[56] Noguchi, M.; Uemura, H.; Naito, S.; Akaza, H.; Yamada, A.; Itoh, K. A phase I study of personalized peptide vaccination using 14 kinds of vaccine in combination with low-dose estramustine in HLA-A24-positive patients with castration-resistant prostate cancer. *Prostate*, **2011**, *71*(5), 470-479.

[57] Terasaki, M.; Shibui, S.; Narita, Y.; Fujimaki, T.; Aoki, T.; Kajiwara, K.; Sawamura, Y.; Kurisu, K.; Mineta, T.; Yamada, A.; Itoh, K. Phase I trial of a personalized peptide vaccine for patients positive for human leukocyte antigen-A24 with recurrent or progressive glioblastoma multiforme. *J. Clin. Oncol.*, **2011**, *29*(3), 337-344.

[58] Noguchi, M.; Kobayashi, K.; Suetsugu, N.; Tomiyasu, K.; Suekane, S.; Yamada, A.; Itoh, K.; Noda, S. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. *Prostate*, **2003**, *57*(1), 80-92.

[59] Noguchi, M.; Itoh, K.; Suekane, S.; Yao, A.; Suetsugu, N.; Katagiri, K.; Yamada, A.; Yamana, H.; Noda, S. Phase I trial of patient-oriented vaccination in HLA-A2-positive patients with metastatic hormone-refractory prostate cancer. *Cancer Sci.*, **2004**, *95*(1), 77-84.

[60] Noguchi, M.; Mine, T.; Yamada, A.; Obata, Y.; Yoshida, K.; Mizoguchi, J.; Harada, M.; Suekane, S.; Itoh, K.; Matsuoka, K. Combination therapy of personalized peptide vaccination and low-dose estramustine phosphate for metastatic hormone refractory prostate cancer patients: an analysis of prognostic factors in the treatment. *Oncol. Res.*, **2007**, *16*(7), 341-349.

[61] Noguchi, M.; Kakuma, T.; Uemura, H.; Nasu, Y.; Kumon, H.; Hirao, Y.; Moriya, F.; Suekane, S.; Matsuoka, K.; Komatsu, N.; Shichijo, S.; Yamada, A.; Itoh, K. A randomized phase II trial of personalized peptide vaccine plus low dose estramustine phosphate (EMP) versus standard dose EMP in patients with castration resistant prostate cancer. *Cancer Immunol. Immunother.*, **2010**, *59*(7), 1001-1009.

[62] Noguchi, M.; Moriya, F.; Suekane, S.; Matsuoka, K.; Arai, G.; Matsueda, S.; Sasada, T.; Yamada, A.; Itoh, K. Phase II study of personalized peptide vaccination for castration-resistant prostate cancer patients who failed in docetaxel-based chemotherapy. *Prostate*, **2012**, *72*(8), 834-845.

[63] Yanagimoto, H.; Mine, T.; Yamamoto, K.; Satoi, S.; Terakawa, N.; Takahashi, K.; Nakahara, K.; Honma, S.; Tanaka, M.; Mizoguchi, J.; Yamada, A.; Oka, M.; Kamiyama, Y.; Itoh, K.; Takai, S. Immunological evaluation of personalized peptide vaccination with gemcitabine for pancreatic cancer. *Cancer Sci.*, **2007**, *98*(4), 605-611.

[64] Yanagimoto, H.; Shiomi, H.; Satoi, S.; Mine, T.; Toyokawa, H.; Yamamoto, T.; Tani, T.; Yamada, A.; Kwon, A.H.; Komatsu, N.; Itoh, K.; Noguchi, M. A phase II study of personalized peptide vaccination combined with gemcitabine for non-resectable pancreatic cancer patients. *Oncol. Rep.*, **2010**, *24*(3), 795-801.

[65] Burris, H.A.; Moore, M.J.; Andersen, J.; Green, M.R.; Rothenberg, M.L.; Modiano, M.R.; Cripps, M.C.; Portenoy, R.K.; Storniolo, A.M.; Tarassoff, P.; Nelson, R.; Dorr, F.A.; Stephens, C.D.; Von Hoff, D.D. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J. Clin. Oncol.*, **1997**, *15*(6), 2403-2413.

[66] Yoshitomi, M.; Yutani, S.; Matsueda, S.; Ioji, T.; Komatsu, N.; Shichijo, S.; Yamada, A.; Itoh, K.; Sasada, T.; Kinoshita, H. Personalized peptide vaccination for advanced biliary tract cancer: IL-6, nutritional status and pre-existing antigen-specific immunity

as possible biomarkers for patient prognosis. *Exp. Ther. Med.*, **2012**, *3*(3), 463-469.

[67] Sato, Y.; Shomura, H.; Maeda, Y.; Mine, T.; Une, Y.; Akasaka, Y.; Kondo, M.; Takahashi, S.; Shinohara, T.; Katagiri, K.; Sato, M.; Okada, S.; Matsui, K.; Yamada, A.; Yamana, H.; Itoh, K.; Todo, S. Immunological evaluation of peptide vaccination for patients with gastric cancer based on pre-existing cellular response to peptide. *Cancer Sci.*, **2003**, *94*(9), 802-808.

[68] Sato, Y.; Maeda, Y.; Shomura, H.; Sasatomi, T.; Takahashi, M.; Une, Y.; Kondo, M.; Shinohara, T.; Hida, N.; Katagiri, K.; Sato, K.; Sato, M.; Yamada, A.; Yamana, H.; Harada, M.; Itoh, K.; Todo, S. A phase I trial of cytotoxic T-lymphocyte precursor-oriented peptide vaccines for colorectal carcinoma patients. *Br. J. Cancer*, **2004**, *90*(7), 1334-1342.

[69] Sato, Y.; Fujiwara, T.; Mine, T.; Shomura, H.; Homma, S.; Maeda, Y.; Tokunaga, N.; Ikeda, Y.; Ishihara, Y.; Yamada, A.; Tanaka, N.; Itoh, K.; Harada, M.; Todo, S. Immunological evaluation of personalized peptide vaccination in combination with a 5-fluorouracil derivative (TS-1) for advanced gastric or colorectal carcinoma patients. *Cancer Sci.*, **2007**, *98*(7), 1113-1119.

[70] Hattori, T.; Mine, T.; Komatsu, N.; Yamada, A.; Itoh, K.; Shiozaki, H.; Okuno, K. Immunological evaluation of personalized peptide vaccination in combination with UFT and UZEL for metastatic colorectal carcinoma patients. *Cancer Immunol. Immunother.*, **2009**, *58*(11), 1843-1852.

[71] Yoshiyama, K.; Terazaki, Y.; Matsueda, S.; Shichijo, S.; Noguchi, M.; Yamada, A.; Mine, T.; Ioji, T.; Itoh, K.; Shirouzu, K.; Sasada, T.; Takamori, S. Personalized peptide vaccination in patients with refractory non-small cell lung cancer. *Int. J. Oncol.*, **2012**, *40*(5), 1492-1500.

[72] Terazaki, Y.; Yoshiyama, K.; Matsueda, S.; Watanabe, N.; Kawahara, A.; Naito, Y.; Suekane, S.; Komatsu, N.; Ioji, T.; Yamada, A.; Mine, T.; Terasaki, M.; Itoh, K.; Takamori, S.; Sasada, T. Immunological evaluation of personalized peptide vaccination in refractory small cell lung cancer. *Cancer Sci.*, **2012**, *103*(4), 638-644.

[73] Matsumoto, K.; Noguchi, M.; Satoh, T.; Tabata, K.; Fujita, T.; Iwamura, M.; Yamada, A.; Komatsu, N.; Baba, S.; Itoh, K. A phase I study of personalized peptide vaccination for advanced urothelial carcinoma patients who failed treatment with methotrexate, vinblastine, adriamycin and cisplatin. *BJU Int.*, **2011**, *108*(6), 831-838.

[74] Suekane, S.; Nishitani, M.; Noguchi, M.; Komohara, Y.; Kokubu, T.; Naitoh, M.; Honma, S.; Yamada, A.; Itoh, K.; Matsuoka, K.; Kanayama, H. Phase I trial of personalized peptide vaccination for cytokine-refractory metastatic renal cell carcinoma patients. *Cancer Sci.*, **2007**, *98*(12), 1965-1968.

[75] Sasada, T.; Komatsu, N.; Suekane, S.; Yamada, A.; Noguchi, M.; Itoh, K. Overcoming the hurdles of randomised clinical trials of therapeutic cancer vaccines. *Eur. J. Cancer*, **2010**, *46*(9), 1514-1519.

[76] Eggermont, A.M. Therapeutic vaccines in solid tumours: can they be harmful? *Eur. J. Cancer*, **2009**, *45*(12), 2087-2090.

[77] Disis, M.L. Immunologic biomarkers as correlates of clinical response to cancer immunotherapy. *Cancer Immunol. Immunother.*, **2011**, *60*(3), 433-442.

[78] Hoos, A.; Eggermont, A.M.; Janetzki, S.; Hodi, F.S.; Ibrahim, R.; Anderson, A.; Humphrey, R.; Blumenstein, B.; Old, L.; Wolchok, J. Improved endpoints for cancer immunotherapy trials. *J. Natl. Cancer Inst.*, **2010**, *102*(18), 1388-1397.

[79] Amos, S.M.; Duong, C.P.; Westwood, J.A.; Ritchie, D.S.; Junghans, R.P.; Darcy, P.K.; Kershaw, M.H. Autoimmunity associated with immunotherapy of cancer. *Blood*, **2011**, *118*(3), 499-509.

[80] López, M.N.; Pereda, C.; Segal, G.; Muñoz, L.; Aguilera, R.; González, F.E.; Escobar, A.; Ginesta, A.; Reyes, D.; González, R.; Mendoza-Naranjo, A.; Larrondo, M.; Compán, A.; Ferrada, C.; Salazar-Onfray, F. Prolonged survival of dendritic cell-vaccinated melanoma patients correlates with tumor-specific delayed type IV hypersensitivity response and reduction of tumor growth factor beta-expressing T cells. *J. Clin. Oncol.*, **2009**, *27*(6), 945-952.

[81] Komatsu, N.; Matsueda, S.; Tashiro, K.; Ioji, T.; Shichijo, S.; Noguchi, M.; Yamada, A.; Doi, A.; Suekane, S.; Moriya, F.; Matsuoka, K.; Kuhara, S.; Itoh, K.; Sasada, T. Gene expression profiles in peripheral blood as a biomarker in cancer patients receiving peptide vaccination. *Cancer*, **2012**, *118*(12), 3208-3221.

[82] Sansone, P.; Bromberg, J. Targeting the interleukin-6/Jak/stat pathway in human malignancies. *J. Clin. Oncol.*, **2012**, *30*(9), 1005-1014.

[83] Nishimoto, N.; Kishimoto, T. Interleukin 6: from bench to bedside. *Nat. Clin. Pract. Rheumatol.*, **2006**, *2*(11), 619-626.

[84] Kuball, J.; de Boer, K.; Wagner, E.; Wattad, M.; Antunes, E.; Weeratna, R.D.; Vicari, A.P.; Lotz, C.; van Dorp, S.; Hol, S.; Greenberg, P.D.; Heit, W.; Davis, H.L.; Theobald, M. Pitfalls of vaccinations with WT1-, Proteinase3- and MUC1-derived peptides in combination with MontanideISA51 and CpG7909. *Cancer Immunol. Immunother.*, **2011**, *60*(2), 161-171.

[85] Krug, L.M.; Dao, T.; Brown, A.B.; Maslak, P.; Travis, W.; Bekele, S.; Korontsvit, T.; Zakhaleva, V.; Wolchok, J.; Yuan, J.; Li, H.; Tyson, L.; Scheinberg, D.A. WT1 peptide vaccinations induce CD4 and CD8 T cell immune responses in patients with mesothelioma and non-small cell lung cancer. *Cancer Immunol. Immunother.*, **2010**, *59*(10), 1467-1479.

[86] Barve, M.; Bender, J.; Senzer, N.; Cunningham, C.; Greco, F.A.; McCune, D.; Steis, R.; Khong, H.; Richards, D.; Stephenson, J.; Ganesa, P.; Nemunaitis, J.; Ishioka, G.; Pappen, B.; Nemunaitis, M.; Morse, M.; Mills, B.; Maples, P.B.; Sherman, J.; Nemunaitis, J.J. Induction of immune responses and clinical efficacy in a phase II trial of IDM-2101, a 10-epitope cytotoxic T-lymphocyte vaccine, in metastatic non-small-cell lung cancer. *J. Clin. Oncol.*, **2008**, *26*(27), 4418-4425.

[87] Slingluff, C.L.; Petroni, G.R.; Chianese-Bullock, K.A.; Smolkin, M.E.; Ross, M.I.; Haas, N.B.; von Mehren, M.; Grosh, W.W. Randomized multicenter trial of the effects of melanoma-associated helper peptides and cyclophosphamide on the immunogenicity of a multipeptide melanoma vaccine. *J. Clin. Oncol.*, **2011**, *29*(21), 2924-2932.

[88] Feyerabend, S.; Stevanovic, S.; Gouttefangeas, C.; Wernet, D.; Hennenlotter, J.; Bedke, J.; Dietz, K.; Pascolo, S.; Kuczyk, M.; Rammensee, H.G.; Stenzl, A. Novel multi-peptide vaccination in Hla-A2+ hormone sensitive patients with biochemical relapse of prostate cancer. *Prostate*, **2009**, *69*(9), 917-927.

[89] Walter, S.; Weinschenk, T.; Stenzl, A.; Zdrojowy, R.; Pluzanska, A.; Szczylik, C.; Staehler, M.; Brugger, W.; Dietrich, P.Y.; Mendrzyk, R.; Hilf, N.; Schoor, O.; Fritsche, J.; Mahr, A.; Maurer, D.; Vass, V.; Trautwein, C.; Lewandrowski, P.; Flohr, C.; Pohla, H.; Stanczak, J.J.; Bronte, V.; Mandruzzato, S.; Biedermann, T.; Pawelec, G.; Derhovanessian, E.; Yamagishi, H.; Miki, T.; Hongo, F.; Takaha, N.; Hirakawa, K.; Tanaka, H.; Stevanovic, S.; Frisch, J.; Mayer-Mokler, A.; Kirner, A.; Rammensee, H.G.; Reinhardt, C.; Singh-Jasuja, H. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat. Med.*, **2012**, *18*(8), 1254-1261.

[90] Meyer, R.G.; Korn, S.; Micke, P.; Becker, K.; Huber, C.; Wölfel, T.; Buhl, R. An open-label, prospective phase I/II study evaluating the immunogenicity and safety of a ras peptide vaccine plus GM-CSF in patients with non-small cell lung cancer. *Lung Cancer*, **2007**, *58*(1), 88-94.

[91] Morse, M.A.; Secord, A.A.; Blackwell, K.; Hobeika, A.C.; Sinnathamby, G.; Osada, T.; Hafner, J.; Philip, M.; Clay, T.M.; Lyerly, H.K.; Philip, R. MHC class I-presented tumor antigens identified in ovarian cancer by immunoproteomic analysis are targets for T-cell responses against breast and ovarian cancer. *Clin. Cancer Res.*, **2011**, *17*(10), 3408-3419.

[92] Holmes, J.P.; Benavides, L.C.; Gates, J.D.; Carmichael, M.G.; Hueman, M.T.; Mittendorf, E.A.; Murray, J.L.; Amin, A.; Craig, D.; von Hofe, E.; Ponniah, S.; Peoples, G.E. Results of the first phase I clinical trial of the novel II-key hybrid preventive HER-2/neu peptide (AE37) vaccine. *J. Clin. Oncol.*, **2008**, *26*(20), 3426-3433.

[93] Perez, S.A.; Kallinteris, N.L.; Bisias, S.; Tzonis, P.K.; Georgakopoulou, K.; Varla-Leftherioti, M.; Papamichail, M.; Thanos, A.; von Hofe, E.; Baxevanis, C.N. Results from a phase I clinical study of the novel Ii-Key/HER-2/neu(776-790) hybrid peptide vaccine in patients with prostate cancer. *Clin. Cancer Res.*, **2010**, *16*(13), 3495-3506.

[94] Kallinteris, N.L.; Lu, X.; Blackwell, C.E.; von Hofe, E.; Humphreys, R.E.; Xu, M. Ii-Key/MHC class II epitope hybrids: a strategy that enhances MHC class II epitope loading to create more potent peptide vaccines. *Expert Opin. Biol. Ther.*, **2006**, *6*(12), 1311-1321.

[95] Takahashi, N.; Ohkuri, T.; Homma, S.; Ohtake, J.; Wakita, D.; Togashi, Y.; Kitamura, H.; Todo, S.; Nishimura, T. First clinical

trial of cancer vaccine therapy with artificially synthesized helper/ killer-hybrid epitope long peptide of MAGE-A4 cancer antigen. *Cancer Sci.*, **2012**, *103*(1), 150-153.

[96] Zandvliet, M.L.; Kester, M.G.; van Liempt, E.; de Ru, A.H.; van Veelen, P.A.; Griffioen, M.; Guchelaar, H.J.; Falkenburg, J.H.; Meij, P. Efficiency and mechanism of antigen-specific CD8+ T-cell activation using synthetic long peptides. *J. Immunother.*, **2012**, *35*(2), 142-153.

[97] Speetjens, F.M.; Kuppen, P.J.; Welters, M.J.; Essahsah, F.; Voet van den Brink, A.M.; Lantrua, M.G.; Valentijn, A.R.; Oostendorp, J.; Fathers, L.M.; Nijman, H.W.; Drijfhout, J.W.; van de Velde, C.J.; Melief, C.J.; van der Burg, S.H. Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. *Clin. Cancer Res.*, **2009**, *15*(3), 1086-1095.

[98] Vermeij, R.; Leffers, N.; Hoogeboom, B.N.; Hamming, I.L.; Wolf, R.; Reyners, A.K.; Molmans, B.H.; Hollema, H.; Bart, J.; Drijfhout, J.W.; Oostendorp, J.; van der Zee, A.G.; Melief, C.J.; van der Burg, S.H.; Daemen, T.; Nijman, H.W. Potentiation of a p53-SLP vaccine by cyclophosphamide in ovarian cancer: a single-arm phase II study. *Int. J. Cancer*, **2012**, *131*(5), E670-680.

[99] Zeestraten, E.C.; Speetjens, F.M.; Welters, M.J.; Saadatmand, S.; Stynenbosch, L.F.; Jongen, R.; Kapiteijn, E.; Gelderblom, H.; Nijman, H.W.; Valentijn, A.R.; Oostendorp, J.; Fathers, L.M.; Drijfhout, J.W.; van de Velde, C.J.; Kuppen, P.J.; van der Burg, S.H.; Melief, C.J. Addition of interferon-α to the p53-SLP® vaccine results in increased production of interferon-γ in vaccinated colorectal cancer patients: A phase I/II clinical trial. *Int. J. Cancer*, **2013**, *132*(7), 1581-1591.

[100] Kakimi, K.; Isobe, M.; Uenaka, A.; Wada, H.; Sato, E.; Doki, Y.; Nakajima, J.; Seto, Y.; Yamatsuji, T.; Naomoto, Y.; Shiraishi, K.; Takigawa, N.; Kiura, K.; Tsuji, K.; Iwatsuki, K.; Oka, M.; Pan, L.; Hoffman, E.W.; Old, L.J.; Nakayama, E. A phase I study of vaccination with NY-ESO-1f peptide mixed with Picibanil OK-432 and Montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen. *Int. J. Cancer*, **2011**, *129*(12), 2836-2846.

[101] Berzofsky, J.A.; Terabe, M.; Oh, S.; Belyakov, I.M.; Ahlers, J.D.; Janik, J.E.; Morris, J.C. Progress on new vaccine strategies for the immunotherapy and prevention of cancer. *J. Clin. Invest.*, **2004**, *113*(11), 1515-1525.

[102] Pulendran, B.; Ahmed, R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell*, **2006**, *124*(4), 849-863.

[103] Steinman, R.M.; Hawiger, D.; Nussenzweig, M.C. Tolerogenic dendritic cells. *Annu. Rev. Immunol.*, **2003**, *21*, 685-711.

[104] Banchereau, J.; Palucka, A.K. Dendritic cells as therapeutic vaccines against cancer. *Nat. Rev. Immunol.*, **2005**, *5*(4), 296-306.

[105] Gilboa, E. DC-based cancer vaccines. *J. Clin. Invest.*, **2007**, *117*(5), 1195-1203.

[106] Reddy, S.T.; Swartz, M.A.; Hubbell, J.A. Targeting dendritic cells with biomaterials: developing the next generation of vaccines. *Trends Immunol.*, **2006**, *27*(12), 573-579.

[107] Tacken, P.J.; de Vries, I.J.; Torensma, R.; Figdor, C.G. Dendritic-cell immunotherapy: from ex *vivo* loading to *in vivo* targeting. *Nat. Rev. Immunol.*, **2007**, *7*(10), 790-802.

[108] Ferrari, M.; Downing, G. Medical nanotechnology: shortening clinical trials and regulatory pathways? *BioDrugs*, **2005**, *19*(4), 203-210.

[109] Almeida, A.J.; Souto, E. Solid lipid nanoparticles as a drug delivery system for peptides and proteins. *Adv. Drug Deliv. Rev.*, **2007**, *59*(6), 478-490.

[110] Vasir, J.K.; Labhasetwar, V. Biodegradable nanoparticles for cytosolic delivery of therapeutics. *Adv. Drug Deliv. Rev.*, **2007**, *59*(8), 718-728.

[111] Langer, R.; Cleland, J.L.; Hanes, J. New advances in microsphere-based single-dose vaccines. *Adv. Drug Deliv. Rev.*, **1997**, *28*(1), 97-119.

[112] Shen, H.; Ackerman, A.L.; Cody, V.; Giodini, A.; Hinson, E.R.; Cresswell, P.; Edelson, R.L.; Saltzman, W.M.; Hanlon, D.J. Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. *Immunology*, **2006**, *117*(1), 78-88.

[113] Zhang, Z.; Tongchusak, S.; Mizukami, Y.; Kang, Y.J.; Ioji, T.; Touma, M.; Reinhold, B.; Keskin, D.B.; Reinherz, E.L.; Sasada, T. Induction of anti-tumor cytotoxic T cell responses through PLGA-nanoparticle mediated antigen delivery. *Biomaterials*, **2011**, *32*(14), 3666-3678.

[114] Noguchi, M.; Itoh, K.; Suekane, S.; Morinaga, A.; Sukehiro, A.; Suetsugu, N.; Katagiri, K.; Yamada, A.; Noda, S. Immunological monitoring during combination of patient-oriented peptide vaccination and estramustine phosphate in patients with metastatic hormone refractory prostate cancer. *Prostate*, **2004**, *60*(1), 32-45.

[115] Noguchi, M.; Itoh, K.; Yao, A.; Mine, T.; Yamada, A.; Obata, Y.; Furuta, M.; Harada, M.; Suekane, S.; Matsuoka, K. Immunological evaluation of individualized peptide vaccination with a low dose of estramustine for HLA-A24+ HRPC patients. *Prostate*, **2005**, *63*(1), 1-12.

[116] Noguchi, M.; Yao, A.; Harada, M.; Nakashima, O.; Komohara, Y.; Yamada, S.; Itoh, K.; Matsuoka, K. Immunological evaluation of neoadjuvant peptide vaccination before radical prostatectomy for patients with localized prostate cancer. *Prostate*, **2007**, *67*(9), 933-942.

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A phase II study of a personalized peptide vaccination for chemotherapy-resistant advanced pancreatic cancer patients

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Abstract. Pancreatic cancer is one of the most aggressive cancers with a median survival time (MST) of <6 months in chemotherapy-resistant patients. Therefore, the development of novel treatment modalities is needed. In the present study, a phase II study of personalized peptide vaccination (PPV) was conducted, in which vaccine antigens were selected and administered based on the pre-existing IgG responses to 31 different pooled peptides, for 41 chemotherapy-resistant advanced pancreatic cancer patients. No vaccine-related severe adverse events were observed. IgG responses specific to at least one of the vaccine peptides were augmented in 14 of 36 patients (39%) and in 18 of 19 patients (95%) tested after the 5th and 11th vaccination, respectively. MST from the first vaccination was 7.9 months with a 1-year survival rate of 26.8%. Higher serum amyloid A (SAA) and C-reactive protein (CRP) levels in pre-vaccination plasma were unfavorable factors for overall survival (OS). Due to the safety profile and the potential clinical efficacy, the conduction of additional clinical trials of PPV for chemotherapy-resistant advanced pancreatic cancer patients is warranted.

Introduction

Pancreatic cancer, the fourth leading cause of cancer-related mortality worldwide, constitutes one of the most aggressive types of cancer (1). There have been substantial advances in the therapeutic modalities for advanced pancreatic cancer, including carbon beam ion radiotherapy (2), systemic chemotherapies using gemcitabine (GEM), tegaful-gimeracil-oteracil potassium (S-1) (3) and oxaliplatin, irinotecan, fluorouracil, leucovorin (Folfirinox) (4), as well as an EGFR-inhibitor erlotinib (5). However, despite these advances, the median survival time (MST) of advanced pancreatic cancer patients from the first or second line of chemotherapy still remains approximately 7-11 (1-5) or 4-6 months (2,6), respectively. Therefore, the development of novel therapeutic approaches including cancer vaccines is needed.

We previously devised a new regimen of peptide-based vaccination, named personalized peptide vaccination (PPV), in which vaccine antigens were selected from 31 different pooled peptides, and administered based on both HLA-class IA types and levels of peptide-specific IgG responses before vaccination (7-10). In our previous clinical trials, immune responses triggered by PPV were well-associated with overall survival (OS) in advanced pancreatic cancer patients under PPV in combination with GEM as the first-line therapy (7,8). GEM did not inhibit immune responses induced by PPV. Furthermore, the MST of advanced pancreatic cancer patients with positive (n=10) or negative (n=8) immune responses was 15.5 and 6 months, respectively, when non-resectable pancreatic cancer patients were treated with PPV and GEM as the first-line therapy. However, there is no trial of PPV for chemotherapy-resistant advanced pancreatic cancer currently available. Consequently, in the present study, a phase II study of PPV in chemotherapy-resistant advanced pancreatic cancer patients was performed.

Materials and methods

Patients. Patients pathologically and/or clinically diagnosed with pancreatic cancer were eligible for inclusion in the present study, when they had failed at least first-line chemotherapy and showed positive IgG responses to at least 2 of the 31 different vaccine candidate peptides as previously reported (10). Additional inclusion criteria were the following: age between 20 and 80 years, Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, positive status for the HLA-A2, -A24, -A3 supertype (A3, A11, A31 or A33) or -A26, life expectancy of at least 12 weeks, and adequate

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hematologic, hepatic and renal function. Exclusion criteria included pulmonary, cardiac or other systemic diseases, acute infection, a history of severe allergic reactions, pregnancy or nursing, and other inappropriate conditions for enrollment as judged by clinicians. The protocol was approved by the Kurume University Ethics Committee, and was registered in the UMIN Clinical Trials Registry (UMIN #08167). After a full explanation of the protocol, a written informed consent was obtained from all the patients prior to enrollment.

Clinical protocol. This was an open-label phase II study, in which the main objectives were to evaluate safety and to address whether PPV in combination with additional chemotherapeutic regimens for chemotherapy-resistant pancreatic cancer patients prolongs MST. Thirty-one peptides, the safety and immunological effects of which were reported in previous clinical studies (8-11), were employed for vaccination [12 peptides for HLA-A2, 14 for HLA-A24, 9 for HLA-A3 supertype (A3, A11, A31 or A33) and 4 for HLA-A26]. The peptides were prepared under the conditions of Good Manufacturing Practice (GMP) by PolyPeptide Laboratories (San Diego, CA, USA) and the American Peptide Company (Vista, CA, USA).

The peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, by assessing the titers of IgG specific to each of the 31 different vaccine candidates (10). A maximum of 4 peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, in complex with incomplete Freund's adjuvant (Montanide ISA 51; Seppic, Paris, France) were subcutaneously administered once a week for 6 consecutive weeks.

After the first cycle of 6 vaccinations, up to 4 vaccine peptides were re-selected according to the titers of peptide-specific IgG and administered every 2 weeks. Vaccine peptides were re-selected at every cycle of 6 vaccinations until the discontinuation of PPV. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0. Complete blood counts and serum biochemical tests were performed at every cycle of 6 vaccinations. The clinical responses were evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST) with radiological findings of computed tomography (CT) scanning or magnetic resonance imaging (MRI) before and after vaccinations.

Measurement of laboratory markers. Levels of C-reactive protein (CRP), serum amyloid A (SAA) and IL-6 in plasma were examined by ELISA using kits from R&D Systems (Minneapolis, MN, USA), Invitrogen (Carlsbad, CA, USA) and eBioscience (San Diego, CA, USA), respectively. Bead-based multiplex assays were used to measure cytokines, including IL-4, IL-13, IL-21, IP-10, BAFF and TGF-β with the Luminex 200 system (Luminex, Austin, TX, USA).

Measurement of immunoglobulins (Igs) reactive to each of the 31 different peptides. The levels of Igs reactive to each of the 31 different peptides were measured using the Luminex 200 system as previously reported (9-11). In brief, plasma was incubated with 100 μl of peptide-coupled color-coded beads for 1.5 h at 30°C, followed by washing and incubation with 100 μl of biotinylated goat anti-human IgG (Vector Laboratories, Burligame, CA, USA). After washing, 100 μl of streptavidin-PE (Invitrogen) was added and incubated for 30 min at 30°C. After washing, the fluorescence on the beads was detected using the Luminex 200 system. The Igs levels were expressed in fluorescence intensity units (FIU) as previously reported (9-11). Peptide-specificity of IgG against each of the 31 peptides was confirmed (unpublished data).

Statistical methods. The Wilcoxon signed-rank test and paired t-test were used to compare differences between pre- and post-vaccination measurements. OS was calculated from the first day of peptide vaccination until the day of death or the last day when the patient was known to be alive. Prognostic factors for OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. Curves for OS were estimated using the Kaplan-Meier method, and the log-rank test was conducted for the comparison of survival curves. Two-sided P-values of <0.05 were considered to indicate statistically significant differences. All statistical analyses were conducted using the JMP version 10.0.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. Between November 2008 and March 2011, 41 advanced pancreatic cancer patients who had failed at least first-line chemotherapy were included in the present study. Patient characteristics are listed in Table I. There were 27 male and 14 female subjects with a median age of 61 years (range, 44-78). All patients had advanced stages of cancer (stage IVa, n=7; IVb, n=24; recurrent, n=10). Prior to enrollment, the patients had failed 1 (n=11), 2 (n=24), 3 (n=5) or 4 (n=1) regimen(s) of chemotherapy. The median duration of chemotherapy prior to PPV was 8 months with a range from 1 to 36 months. The performance status at the time of enrollment was grade 0 (n=37) or 1 (n=4). The numbers of vaccine peptides employed at the first cycle of vaccinations were 4 peptides in 33 patients, 3 in 5 patients and 2 in 3 patients. The median number of vaccinations was 10 with a range of 3 to 36. PPV was combined with GEM (n=11), S-1 (n=6), GEM and S-1 (n=8) or other combinations of chemotherapeutic agents including CDDP-based regimens (n=8). PPV alone was administered to 8 patients, since chemotherapy could not be tolerated (n=4) or due to patient refusal (n=4).

Toxicities. A grade 1 or 2 dermatological reaction at the injection site was observed in 39 cases. Anemia (n=15), lymphocytopenia (n=20), thrombocytopenia (n=11), leukocytopenia (n=7), hypoalbuminemia (n=15) and hyperglycemia (n=8) were also frequently observed. Grade 3 adverse events included anemia (n=1), lymphocytopenia (n=1), hypertension (n=1), GGT increase (n=1) and creatinine increase (n=1). According to assessment by the Independent Safety Evaluation Committee in this trial, all the grade 3 adverse events were concluded to be not directly associated with PPV.

Humoral responses to peptides. IgG responses specific to the vaccine peptides in pre- and post-vaccination plasma samples

Table I. Patient characteristics.

Characteristic	Value
Age (years), median (range)	61 (44-78)
Gender, n	
Male	27
Female	14
Disease location, n	
Head	14
Body	15
Limbs	6
Body and limbs	6
Performance status, n	
0	37
1	4
Stage, n	
IVa	7
IVb	24
Recurrent	10
No. of previous regimens, n	
1	11
2	24
3	5
4	1
Duration of previous treatment (months), median (range)	8 (1-36)
No. of vaccinations, median (range)	10 (3-36)
Combined treatment, n	
(-)	8
GEM	11
S-1	6
GEM and S-1	8
Other regimens	8
Treatment response, n	
SD	28
PD	13
Overall survival time (days), median (95% CI)	238 (151-313)

GEM, gemcitabine; S-1, tegaful-gimeracil-oteracil potassium; SD, stable disease; PD, progressive disease; CI, confidence interval.

were analyzed. Post-vaccination plasma samples were available from 36 and 17 patients after the 5th and 11th vaccination, respectively. When peptide-specific IgG titers to at least one of the vaccine peptides in the post-vaccination plasma were >2-fold higher compared to those in the pre-vaccination plasma, antigen-specific humoral responses were considered to be increased. The IgG responses specific to at least one of the vaccine peptides were augmented in 14 of 36 patients (39%) and in 18 of 19 patients (95%) after the 5th and 11th vaccination, respectively (Table II).

Table II. IgG responses to the vaccinated peptides.

		IgG response		
Patient no.	Peptide	Pre	5th	11th
1	ppMAPkkk-432	43	40	na
	WHSC2-103	68	69	na
	HNRPL-501	191	**638**	na
	HNRPL-140	209	189	na
2	SART3-109	226	**1,896**	na
	Lck-422	44	66	na
	CypB-129	23	45	na
	WHSC2-103	322	401	na
3	PSA-248	28	**4,999**	**28,025**
	MRP3-1293	75	70	**3,259**
	SART2-161	37	38	**7,860**
	Lck-486	38	31	**23,697**
4	MRP3-503	57	56	na
	MRP3-1293	79	69	na
	SART2-161	51	53	na
	Lck-486	53	ND	na
5	CypB-129	161	120	**12,717**
	ppMAPkkk-432	368	ND	ND
	UBE2V-43	396	399	**60,508**
	SART3-302	272	235	**11,267**
	HNRPL-501	150	**343**	ND
6	HNRPL-140	13	ND	na
	SART3-302	40	ND	na
7	SART3-109	42	52	na
	SART3-511	27	ND	na
	Lck-90	13	ND	na
	Lck-449	45	ND	na
8	SART2-93	32	18	na
	PAP-213	1,249	1,573	na
	EGF-R-800	40	ND	na
	MRP3-503	98	38	na
	SART3-109	23	11	na
9	Lck-246	376	623	**3,264**
	UBE2V-43	188	ND	**16,549**
	UBE2V-85	294	314	**2,053**
	SART3-302	207	330	**1,929**
	HNRPL-140	ND	**494**	**2,780**
10	HNRPL-501	578	ND	ND
	UBE2V-85	70	ND	14
	SART3-302	36	ND	ND
	SART3-309	18	ND	ND
11	SART3-109	21	ND	**653**
	MRP3-503	69	ND	**14,787**
	PTHrP-102	14	ND	ND
12	SART2-93	164	ND	na
	Lck-208	206	13	na
	Lck-486	245	298	na
	EZH2-735388	503	na	

Table II. Continued.

Patient no.	Peptide	IgG response Pre	5th	11th
12	Lck-422	783	532	na
	HNRPL-140	456	380	na
13	SART3-109	1,475	1,279	na
	Lck-486	1,644	1,833	na
14	SART3-109	2,309	2,136	**6,782**
	MRP3-1293	43	40	**23,180**
	SART2-161	32	27	ND
	Lck-486	1,515	1,234	**267,768**
15	SART3-109	1,500	**5,872**	**180,917**
	SART2-161	31	22	**3,278**
	Lck-486	650	224	**58,780**
	Lck-488	54	37	**21,889**
	SART3-511	99	57	ND
16	SART3-511	1,699	1,503	1,522
	PAP-248	70	69	ND
	Lck-422	180	ND	16
	WHSC2-103	188	ND	**2,629**
	Lck-90	35	45	63
	CypB-129	16	23	20
17	ppMAPkkk-432	83	88	ND
	SART3-109	62	49	ND
	Lck-486	2,176	2,191	**3,523,034**
	PTHrP-102	129	162	135
	SART2-93	47	**100**	59
18	MRP3-1293	103	ND	na
	Lck-486	5,731	10,510	na
	PSMA-624	99	ND	na
	ppMAPkkk-432	126	115	na
	SART3-109	55	50	na
	Lck-488	38	35	na
19	CypB-129	57	53	na
	ppMAPkkk-432	106	90	na
	HNRPL-501	974	934	na
	SART3-302	473	**2,233**	na
	Lck-246	17	**61**	na
20	Lck-246	409	441	**2,349**
	EGF-R-800	83	134	183
	Lck-486	95	72	**37,353**
	EZH2-735	117	ND	**10,454**
	CypB-129	183	192	190
	ppMAPkkk-432	120	185	233
21	PAP-213	48	**98**	na
	Lck-486	20	22	na
22	CypB-129	109	112	**393**
	Lck-246	22	13	**56**
	WHSC2-141	22	ND	15
	SART3-302	631	**1,459**	**5,168**
	Lck-422	14	12	**78**

Table II. Continued.

Patient no.	Peptide	IgG response Pre	5th	11th
23	PAP-213	13	**123**	**4,179**
	Lck-486	25	**580**	**2,552**
	Lck-449	37	37	43
	WHSC2-103	40	14	**165**
	SART3-511	ND	**289**	173
	PAP-248	ND	**1,200**	63
24	PAP-213	122	122	na
	Lck-449	129	102	na
	CypB-129	186	183	na
	WHSC2-103	69	ND	na
25	PAP-213	16	**2,772**	na
	PSA-248	64	**1,372**	na
	Lck-486	17	**105**	na
26	CypB-129	90	81	105
	Lck-246	20	12	**39**
	SART3-309	12	**374**	**4,738**
	PAP-248	21	ND	ND
27	SART2-93	11	ND	**55**
	SART3-109	156	222	**1,871**
	Lck-486	185	313	**12,511**
	Lck-488	15	12	**3,980**
	PAP-213	ND	**14**	**ND**
28	PAP-213	31	44	**657**
	PSA-248	45	**446**	**15,954**
	EGF-R-800	30	33	**2,926**
	Lck-486	22	23	**11,356**
29	SART2-93	11	11	na
	Lck-486	25	ND	na
	Lck-488	14	16	na
30	CypB-129	246	232	na
	WHSC2-141	317	21	na
	SART3-302	86	**865**	na
	Lck-208	11	**2,016**	na
31	SART2-93	40	37	**478**
	Lck-486	23	32	**2,567**
	Lck-488	31	47	**20,641**
	PTHrP-102	40	46	**523**
32	WHSC2-141	433	398	**20,518**
	PSA-248	29	**2,109**	**13,221**
	MRP3-1293	149	**4,155**	**11,903**
		121		
	Lck-486	121	18,577	
33	SART2-93	22	**51**	60
	SART3-109	14	ND	16
	Lck-486	39	ND	**2,479**
	SART2-161	ND	**76**	59
34	CypB-129	263	239	na
	WHSC2-103	43	ND	na

Table II. Continued.

Patient no.	Peptide	IgG response Pre	5th	11th
34	WHSC2-141	231	125	na
	SART3-734	32	ND	na
35	MRP3-1293	62	ND	na
	Lck-486	85	ND	na
	SART3-734	123	ND	na
	CypB-129	149	93	na
36	SART2-93	13	11	12
	SART3-109	11,200	10,657	10,093
	Lck-488	16	13	**2,017**
	EGF-R-800	ND	**11**	ND

Underlined peptides indicate the selection of new peptides for the second cycle of PPV. Bold values represent increased IgG responses. na, not applicable; ND, no data.

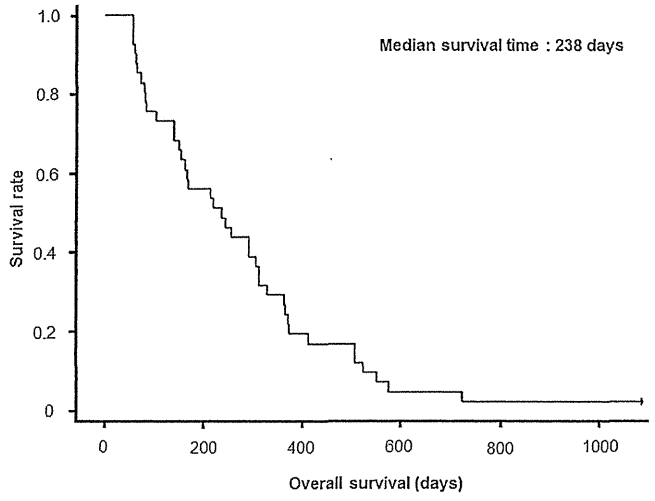

Figure 1. Kaplan-Meier survival analysis of the enrolled patients. The median survival time (MST) of patients who were vaccinated (n=41) was 238 days (7.9 months) and the 1-year survival rate was 26.8%.

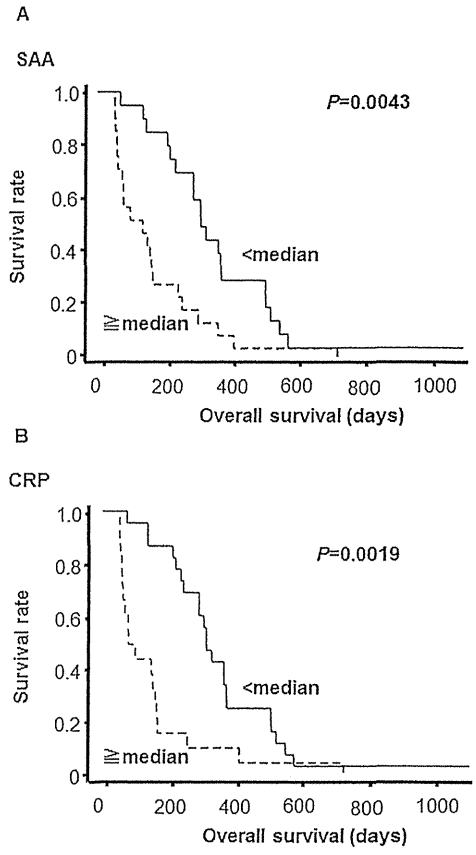

Figure 2. Evaluation of pre-vaccination factors by Kaplan-Meier survival analysis. Patients were divided into two subgroups according to the median value of the (A) SAA and (B) CRP levels before vaccination. Curves for overall survival were estimated by the Kaplan-Meier method, and differences in survival rates were evaluated using the log-rank test. SAA, serum-amyloid A; CRP, C-reactive protein.

Laboratory markers. Two inflammation markers, CRP and SAA, and 7 cytokines including IL-4, IL-6, IL-13, IL-21, IP-10, BAFF and TGF-β, were examined in plasma before and after the 5th vaccination. Since 5 of 41 patients did not complete the first cycle of 6 vaccinations due to rapid disease progression, they were excluded from the marker analysis. However, no significant differences before and after vaccinations were observed in the markers tested (data not shown).

Clinical outcome. No complete response (CR) or partial response (PR) was observed during PPV. Optimum clinical responses after the 6th vaccination or at discontinuation of PPV were observed in 28 cases of stable disease (SD) and 13 cases of progressive disease (PD) (Table I). MST from the first vaccination was 7.9 months (238 days) with a 1-year survival rate of 26.8% (Table I). All the 41 patients, except for 1 patient, had succumbed to the disease at the time of examination. Survival curve is shown in Fig. 1. MST in patients treated with PPV in combination with (n=33) or without (n=8) chemotherapies was 9.6 or 3.1 months, respectively (P=0.0013) (data not shown). When calculated from the initiation of the first-line chemotherapy, MST of all 41 cases was 19.0 months [95% confidence interval (CI), 15.0-25.0 months].

Prognostic factors for OS. Pre-vaccination prognostic biomarkers for OS were investigated in 36 patients who completed at least the first cycle of 6 vaccinations. SAA levels in pre-vaccination samples were found to be inversely associated with OS using the univariate Cox proportional hazards model [hazard ratio (HR) per 1 mg/dl increment = 1.10, 95% CI=1.03-1.15, P=0.004] (Table III). CRP levels also showed a significant association (HR per 1 mg/dl increment = 1.68, 95% CI=1.03-2.58, P=0.039). Similar results were obtained using the multivariate Cox proportional hazards model. The patients were allocated into two subgroups according to the median value of SAA or CRP. The survival curves were estimated by the Kaplan-Meier method and differences in survival rates were compared using the log-rank test. The patients with higher SAA (P=0.0043) or CRP levels (P=0.0019) in the pre-vaccination samples exhibited worse prognosis (Fig. 2).

Table III. Univariate and multivariate analyses with pre-vaccination clinical findings and laboratory data.

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% Cl)	P-value[a]	Hazad ratio (95% Cl)	P-value[a]
Age (years)	1.58 (0.40-6.44)	0.52		
Gender (female<male)	0.98 (0.52-1.95)	0.96		
Clinical stage (IVa<recurrent<IVb)	1.18 (0.78-1.80)	0.43		
Duration of previous chemotherapy (months)	0.98 (0.94-1.02)	0.27		
Regimen no. of previous chemotherapy	0.93 (0.59-1.44)	0.75		
Lymphocyte count ($x10^2/mm^3$)	1.00 (1.00-1.00)	0.39		
Hemoglobin (g/dl)	0.93 (0.75-1.16)	0.53		
Albumin (g/dl)	0.58 (0.32-1.10)	0.09		
Creatinine (mg/dl)	1.88 (0.51-5.23)	0.31		
SAA (mg/dl)	1.09 (1.03-1.15)	0.004[b]	1.08 (0.99-1.18)	0.09
CRP (mg/dl)	1.68 (1.03-2.58)	0.039[b]	0.95 (0.41-2.06)	0.91

[a]P-values determined by Cox proportional hazard regression model; [b]significant difference. Cl, confidence interval; SAA, serum amyloid A; CRP, C-reactive protein.

In addition, concerning post-vaccination samples, the patients with boosted IgG responses (n=19) [in response to the vaccinated (n=14) or unvaccinated peptides selected for the 2nd cycle of PPV (n=5)] exhibited better prognosis compared to those with no IgG boosting (n=17) (P=0.0485) (data not shown).

Discussion

The MST of 41 chemotherapy-resistant advanced pancreatic cancer patients under PPV was 7.9 months with a 1-year survival rate of 26.8%. Among them, the MST in patients treated with PPV combined with (n=33) or without (n=8) chemotherapies was 9.6 or 3.1 months, respectively (P=0.0013). OS of the patients treated with PPV not combined with chemotherapies was significantly short, suggesting that PPV alone did not provide survival benefits to advanced pancreatic cancer patients. This failure was expected based on the results from our previous study (13). These results suggest that PPV has the potential to improve OS in chemotherapy-resistant advanced pancreatic cancer patients when administered in combination with chemotherapeutic agents.

With regard to post-vaccination biomarkers, several factors, including CTL responses, Th1 responses, delayed-type hypersensitivity (DTH) and autoimmunity, have been reported to be associated with clinical responses in some clinical trials (14,15). We have also shown that an increase in peptide-specific IgG and/or CTL responses after PPV is significantly associated with longer OS (11,12). In contrast to such post-vaccination biomarkers, there are currently no validated pre-vaccination prognostic biomarkers widely used. Therefore, this issue was addressed in the present study. As a result, plasma SAA and CRP levels were inversely correlated with OS. These results were expected based on our previous study on PPV (10). These biomarkers are suggested to be important not only in cancer vaccines, but also in other treatment modalities for advanced pancreatic cancers.

Collectively, due to the safety profile and the potential clinical efficacy of PPV, further clinical trials to determine a protocol suitable for PPV-based therapy in chemotherapy-resistant advanced pancreatic cancer patients are warranted.

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References

1. Kindler HL, Ioka T, Richel DJ, *et al*: Axitinib plus gemcitabine versus placebo plus gemcitabine in patients with advanced pancreatic adenocarcinoma: a double-blind randomised phase 3 study. Lancet Oncol 12: 256-262, 2011.
2. Okada T, Kamada T, Tsuji H, *et al*: Carbon ion radiotherapy: clinical experiences at National Institute of Radiological Science (NIRS). J Radiat Res 51: 355-364, 2010.
3. Nakamura K, Yamaguchi T, Ishihara T, Sudo K, Kato H and Saisho H: Phase II trial of oral S-1 combined with gemcitabine in metastatic pancreatic cancer. Br J Cancer 94: 1575-1579, 2006.
4. Conroy T, Desseigne F, Ychou M, *et al*; Groupe Tumeurs Digestives of Unicancer and PRODIGE Intergroup: FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364: 1817-1825, 2011.
5. Moore MJ, Goldstein D, Hamm J, *et al*: Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 25: 1960-1966, 2007.
6. Pelzer U, Schwaner I, Stieler J, *et al*: Best supportive care (BSC) versus oxaliplatin, folinic acid and 5-fluorouracil (OFF) plus BSC in patients for second-line advanced pancreatic cancer: a phase III study from the German CONKO-study group. Eur J Cancer 47: 1676-1681, 2011.
7. Yanagimoto H, Mine T, Yamamoto K, *et al*: Immunological evaluation of personalized peptide vaccination with gemcitabine for pancreatic cancer. Cancer Sci 98: 605-611, 2007.

8. Yanagimoto H, Shiomi H, Satoi S, *et al*: A phase II study of personalized peptide vaccination combined with gemcitabine for non-resectable pancreatic cancer patients. Oncol Rep 24: 795-801, 2010.
9. Terasaki M, Shibui S, Narita Y, *et al*: Phase I trial of a personalized peptide vaccine for patients positive for human leukocyte antigen-A24 with recurrent or progressive glioblastoma multiforme. J Clin Oncol 29: 337-344, 2011.
10. Terazaki Y, Yoshiyama K, Matsueda S, *et al*: Immunological evaluation of personalized peptide vaccination in refractory small cell lung cancer. Cancer Sci 103: 638-644, 2012.
11. Noguchi M, Mine T, Komatsu N, *et al*: Assessment of immunological biomarkers in patients with advanced cancer treated by personalized peptide vaccination. Cancer Biol Ther 10: 1266-1279, 2011.
12. Mine T, Sato Y, Noguchi M, *et al*: Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing, peptide-specific cellular responses. Clin Cancer Res 10: 929-937, 2004.
13. Yamamoto K, Mine T, Katagiri K, *et al*: Immunological evaluation of personalized peptide vaccination for patients with pancreatic cancer. Oncol Rep 13: 874-883, 2005.
14. Lopez MN, Pereda C, Segal G, *et al*: Prolonged survival of dendritic cell-vaccinated melanoma patients correlates with tumor-specific delayed type IV hypersensitivity response and reduction of tumor growth factor beta-expressing T cells. J Clin Oncol 27: 945-952, 2009.
15. Firpo MA, Gay DZ, Granger SR, *et al*: Improved diagnosis of pancreatic adenocarcinoma using haptoglobin and serum amyloid A in a panel screen. World J Surg 33: 716-722, 2009.

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Aldehyde Dehydrogenase 1 Identifies Cells with Cancer Stem Cell-Like Properties in a Human Renal Cell Carcinoma Cell Line

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Abstract

Cancer stem cells (CSC) or cancer stem cell-like cells (CSC-LCs) have been identified in many malignant tumors. CSCs are proposed to be related with drug resistance, tumor recurrence, and metastasis and are considered as a new target for cancer treatment; however, there are only a few reports on CSCs or CSC-LCs in renal cell carcinoma (RCC). Different approaches have been reported for CSC identification, but there are no universal markers for CSC. We used two different approaches, the traditional side population (SP) approach, and the enzymatic (aldehyde dehydrogenase 1 (ALDH1)) approach to identify CSC-LC population in two RCC cell lines, ACHN and KRC/Y. We found that ACHN and KRC/Y contain 1.4% and 1.7% SP cells, respectively. ACHN SP cells showed a higher sphere forming ability, drug resistance, and a slightly higher tumorigenic ability in NOD/SCID mice than Non-SP (NSP) cells, suggesting that cells with CSC-LC properties are included in ACHN SP cells. KRC/Y SP and NSP cells showed no difference in such properties. ALDH1 activity analysis revealed that ACHN SP cells expressed a higher level of activity than NSP cells (SP vs. NSP: 32.7% vs 14.6%). Analysis of ALDH1-positive ACHN cells revealed that they have a higher sphere forming ability, self-renewal ability, tumorigenicity and express higher mRNA levels of CSC-LC property-related genes (e.g., ABC transporter genes, self-replication genes, anti-apoptosis genes, and so forth) than ALDH1-negative cells. Drug treatment or exposure to hypoxic condition induced a 2- to 3-fold increase in number of ALDH1-positive cells. In conclusion, the results suggest that the ALDH1-positive cell population rather than SP cells show CSC-LC properties in a RCC cell line, ACHN.

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Introduction

Renal cell carcinoma (RCC) is one of the most common malignancies of the genitourinary tract, accounting for 116,500 deaths in 2008 according to the World Health Organization [1]. The incidence of RCC has been steadily rising over the past 30 years [2]. Furthermore, because metastatic RCC is notoriously resistant to most conventional therapies, such as chemotherapy and radiotherapy, the prognosis of patients with RCC is poor as one-third of patients already have metastatic disease at the initial diagnosis and 30–40% of them develop distant metastases after resection of the primary tumor [3]. In recent years, the molecular targeted therapies that have been developed have shown significant objective responses [4–6], and they are now recognized as the current standard therapies of metastatic RCC. However, the efficacy of these molecular target therapies is insufficient.

The two dominant models of carcinogenesis are the stochastic model (clonal evolution) and the hierarchic organization of tumor (cancer stem cell (CSC)) model. According to the traditional clonal evolution model, tumor formation is the consequence of accumulating random genetic events in normal differentiated cells, whereas the CSC model postulates that a single CSC gives rise to a hierarchical organization within a tumor [7,8]. Recent studies suggest that CSCs may be responsible for tumorigenesis and contribute to some individuals' resistance to cancer therapy, which resulted in cancer relapse and metastasis [9,10]. Therefore, it is widely believed that identification and characterization of CSC or cancer stem cell-like cell (CSC-LC) may contribute significantly to the development of effective therapies. Bussolati et al. identified a population of CD105 positive tumor initiating cells in RCCs, and reviewed the literature on the role of stem cells in human RCC [11,12]. Kim et al. reported that the expression of stem cell markers, OCT4 and CD133, may serve, respectively, as a poor and favorable prognostic marker, in papillary RCC [13]. In addition, they suggested that the expression of CD133 is a favorable prognostic marker in clear cell RCC [14].

There are many reports that CSC-LCs of some solid tumors are present in side population (SP) cells [15,16], but there are only a few reports on the role of SP cells in human RCC [17,18]. SP cells were originally identified in flow cytometric analyses by their ability to efflux the vital DNA dye, Hoechst 33342, resulting in Hoechst-negative SP cells and Hoechst-positive Non-SP (NSP) cells. Previous studies of cancers in vitro and primary tumors in vivo have shown that SP cells are uniquely capable of

generating both SP and NSP cell populations, exhibiting properties consistent with stem cells or CSC. SP cells express high levels of ATP-binding cassette (ABC) transporter family members, especially ABCG2, and exhibit more chemotherapeutic drug resistance than NSP cells in cell lines derived from some human malignant solid tumors, such as breast cancer, lung cancer, ovarian cancer and squamous cell cancer [19–21].

Recently, it has been reported that aldehyde dehydrogenase 1 (ALDH1) is responsible for the oxidation of retinol to retinoic acid and plays pivotal roles in embryonic development and homeostasis in several organs [22]. Some researchers have reported that high expression of ALDH1 was associated with drug resistance and poor prognosis, and that ALDH1 is a CSC marker [23,24]. Ozbek et al. reported that ALDH1 expression was correlated with tumor grade in RCC [25], but the biological features of ALDH1-positive cells in RCC are still largely unknown.

In this study, we isolated SP cells from two human RCC cell lines and systematically investigated the CSC properties of the SP cells and ALDH1-positive cells, and relationship between SP cells and ALDH1-positive cells.

Materials and Methods

Cell Lines and Animals

We used two RCC cell lines: one derived from malignant pleural effusion of a patient with RCC (ACHN) and the other derived from primary lesion of a patient with RCC (KRC/Y). These 2 RCC cell lines have high proliferative and colony forming abilities *in vitro* and possess high tumorigenicity in even nude mice *in vivo*. ACHN was purchased from American Type Culture Collection. KRC/Y was established in our laboratory [26]. Culture medium for ACHN consisted of modified Eagle's medium (EMEM) (Gibco, BRL/Life Technologies Inc., Gaithersburg, MD, USA). Culture medium for KRC/Y consisted of Dulbecco's modified medium (DMEM) (Nissui Seiyaku Co., Tokyo, Japan) supplemented with heat-inactivated (56°C, 30 min) 5% fetal bovine serum (FBS, Bioserum, Vic, Australia), 100 U/mL penicillin and 100 μg streptomycin (Gibco BRL/Life Technologies Inc.). Cells were cultured in an atmosphere of 5% CO_2 in air at 37°C. Female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (5 week-old) were purchased (Clea Japan, Inc., Osaka, Japan), and housed in laminar-flow cabinets under specific pathogen-free conditions. All procedures were approved by the Ethics Review Committee for Animal Experimentation of Kurume University School of Medicine.

Expression of CSC Markers in RCC Cell Lines

We analyzed the expression of the putative CSC markers ABCG2, CD90, CD105, CD133 and epithelial cell adhesion molecule (EpCAM) in ACHN and KRC/Y. Cells were incubated in the dark at 4°C for 30 minutes with fluorescence-conjugated monoclonal antibodies, including fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD90 antibody (5E10, BD Biosciences, San Jose, CA, USA) and mouse anti-human CD105 antibody (MEM-226, EXBIO, Praha, Czech) and phycoerythrin (PE)-conjugated CD133/2 antibodies (293C3, Miltenyi Biotec, Bergisch-Gladbach, Germany) and anti-EpCAM antibody (EBA-1, BD Biosciences). Cells with mouse anti-BCRP monoclonal antibody (ABCG2) (BXP-21, Chemicon, Temecula, CA, USA) were incubated for 30 minutes and further incubated in the dark at 4°C for 30 minutes with FITC-conjugated goat anti-mouse Ig (FITC-GAM) (BD Biosciences). Cells were washed, resuspended and analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

SP Cell Identification and CSC Marker Expression in SP and NSP Cells

Cultured cells with 80% confluence were detached with accutase (Innovative Cell Technologies, Inc., San Diego, USA) and suspended at 1×10^6 cells/mL in phosphate-buffered saline (PBS) supplemented with 2% FBS and then incubated with Hoechest 33342 dye alone (5 μg/mL for ACHN and 10 μg/mL for KRC/Y) (SIGMA-Aldrich, Saint Louis, MO, USA) or with 20 μg/mL reserpine (SIGMA-Aldrich) at 37°C for 60 min. Samples were washed, centrifuged and resuspended in 2 mL cold PBS supplemented with 2% FBS, then 1 μg/mL propidium iodide (PI) (BD Biosciences) was added and the cells were filtered through a 40 μm cell strainer (BD Biosciences). Flow cytometric analysis was performed as previously described [27]. Reserpine is conventionally used as a guiding parameter to determine the boundary between SP and NSP cells. Analyses were carried out with a FACSAria II (BD Biosciences). The expression of CD90 and EpCAM in ACHN, and that of CD105 and EpCAM in KRC/Y, in SP and NSP cells was further examined. Cells were stained using the method described above.

Cell Growth Assay of SP and NSP Cells

A total of 2,000 SP cells and NSP cells were plated in 96-well plates and cultured in a CO_2 incubator. The cells were harvested at 24, 48, 72, 96, 120 or 144 hours and the proliferation was examined in colorimetric assays using 3-(4,5-dimethylthiazol-2yl-yl-)-2,5-diphenyl tetrazolium bromide (MTT) cell growth assay kits (Chemicon, Temecula, CA, USA) as described elsewhere [28].

Colony Formation Assay of SP and NSP Cells

The soft agar anchorage independent clonogenic growth assay was performed. Briefly, 2×10^4 cells were suspended in 2 mL of EMEM or DMEM containing 0.36% soft agar (Gibco BRL/Life Technologies Inc.) and 10% FBS in a 35 mm dish. The cell suspension was then overlaid on a presolidified 0.72% hard agar. The medium containing 0.36% soft agar was supplemented once a week. Colonies (>10 cells) that arose within 3 weeks were presented as clonogenicity. Five dishes were examined for each cell type and blindly counted under the microscope (×200) in all fields.

Sphere Formation Assay of SP and NSP Cells

Isolated SP and NSP cells from the two cell lines (4,000 cells/dish) were cultured in serum-free medium including 10 ng/mL epidermal growth factor (EGF) (Sankojunyaku, Tokyo, Japan) and 20 ng/mL basic fibroblast growth factor (bFGF) (Sankojunyaku) using ultra-low-attachment 6-well plates (Corning Inc., Corning, NY, USA) for 1 week, after which sphere formation was assessed by counting the number of spheres (>3 cells) under microscope (×200).

Drug Resistance Assay

Isolated SP and NSP cells were planted at 2,000 cells per well in 96-well plates, and the effect of the multikinase inhibitor Sorafenib (2 μM) (Cell Signaling Technology. Inc., Danvers, MA, USA) and IFNα (4,000 IU/mL) (OIF, Otsuka Pharma Co., Ltd., Tokyo, Japan) was examined. Drug resistance was determined after treatment for 72, 96 or 144 hours by MTT assay.

Tumorigenicity Assays of SP and NSP Cells in vivo

To explore tumorigenic capacity, SP and NSP cells (1, 10 or 100×10^3) were isolated from the two RCC cell lines, placed in 100 μL medium, and separately injected into the subcutaneous